# THE HISTOPATHOLOGICAL DIAGNOSIS OF MYELODYSPLASTIC SYNDROMES AND ACUTE NONLYMPHOBLASTIC LEUKAEMIA USING GLYCOL METHACRYLATE EMBEDDED BONE MARROW BIOPSIES

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# CHAPTER ONE

## INTRODUCTION AND OBJECTIVES

The myelodysplastic syndromes [MDS] are clonal disorders of haematopoiesis characterized by an irreversible derangement in the development of the haematopoietic cell lines. They are currently thought to arise from mutational events in early stem cell development, (1,2,3,4) and it has been suggested that progression to acute leukaemia involves a further stem cell mutation (2,3,5). *De novo* acute leukaemia [AL] is a neoplastic proliferation of immature haemopoietic cells in the marrow which arises after the malignant transformation of a single haemopoietic stem cell (2,4,6).

The diagnosis of MDS and acute non-lymphoblastic leukaemia [ANLL] is made by careful examination of blood films and bone marrow aspirates stained with appropriate dyes (2,4,6,7,8,9,10,11,12).

Because aspirates are diluted with sinusoidal blood, they may not accurately reflect the changes that are inherent in the myelodysplastic disorder and leukaemic process (13). On the other hand, a long core of marrow obtained by trephine biopsy provides a large volume of tissue, and overcomes the problems associated with aspiration due to markedly hypercellular infiltration or increased reticulin fibrosis (14,15,16,17). In such a situation, trephine imprints may provide some limited information.

Paraffin-embedded bone marrow trephines, which are the most widely used for diagnostic purposes, need to be decalcified prior to sectioning. This has a number of effects, and results in interference with the evaluation of the bone marrow (shrinkage, reduction in the quality of staining of the cells, and an impaired ability to apply the special stains used in marrow evaluation) (15,18,19).

On the other hand, glycol methacrylate [GMA]-embedding of marrow cores, which obviates the need for decalcification, shows structural organization of the marrow,

gives excellent cellular detail, and allows the application of cytochemical and immunocytochemical staining (13,14,20,21,22,23,24,25,26).

In order to document the extent to which the quantitative and qualitative morphological criteria proposed by the French-American-British [FAB] Cooperative Group could be applied directly to GMA-embedded marrow biopsy specimens, haematological studies were conducted on 13 newly diagnosed MDS patients and on 16 newly diagnosed ANLL patients. All cases were diagnosed and subsequently treated in the Department of Haematology at Groote Schuur Hospital.

Further objectives were to assess the degree of diagnostic agreement between GMA-embedded marrow biopsies and marrow smears, to establish whether cytochemistry and immunophenotyping could be applied to these sections and to assess if such techniques could be helpful in subdivision of MDS and ANLL.

# CHAPTER TWO

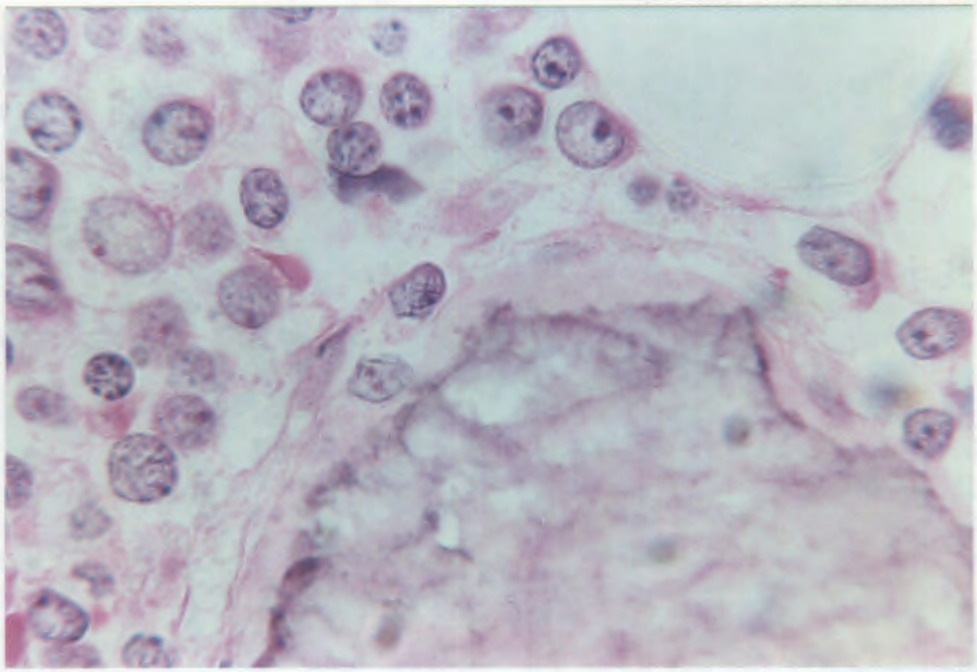
## LITERATURE REVIEW

The diagnosis of ANLL and MDS is based primarily on the morphological and cytochemical characteristics of both the peripheral blood film and a sample of marrow obtained by aspiration. However aspirate samples are small, often selective and diluted with blood. As a result, some cell populations may not be represented in the aspirate smear but can be seen in the marrow biopsy sections (13).

Cells which are firmly anchored within the marrow because of increased reticulin or those which are focally distributed may not be withdrawn on aspiration and thus may be excluded from examination (1,13,27,28,29,30,31).

One of the main advantages of bone marrow biopsy in haematological investigations is that it is possible to study haemopoietic cells in the marrow cavities (13,28,29,30,32,33,34) [Fig.1].

**Figure 1:** **Normocellular marrow with well preserved marrow architecture and intact interface between marrow and bone. [H&Ex400] GMA**



Normally, marrow erythroid precursors are present intertrabecularly in small clusters, in different stages of maturation. Early myeloid precursors are in proximity to the endosteal surfaces of trabeculae and to arterial vessels, whereas mature forms are scattered throughout the central intertrabecular areas. A distortion of this topographical distribution is found in several diseases, but typically in MDS (28,29,30,32).

Differentiation of some cases of MDS and ANLL may be difficult in decalcified, paraffin-embedded marrow biopsy specimens because of the reported inability to obtain reproducible blast counts, (31) and because a considerable percentage of cells cannot be easily recognized (34).

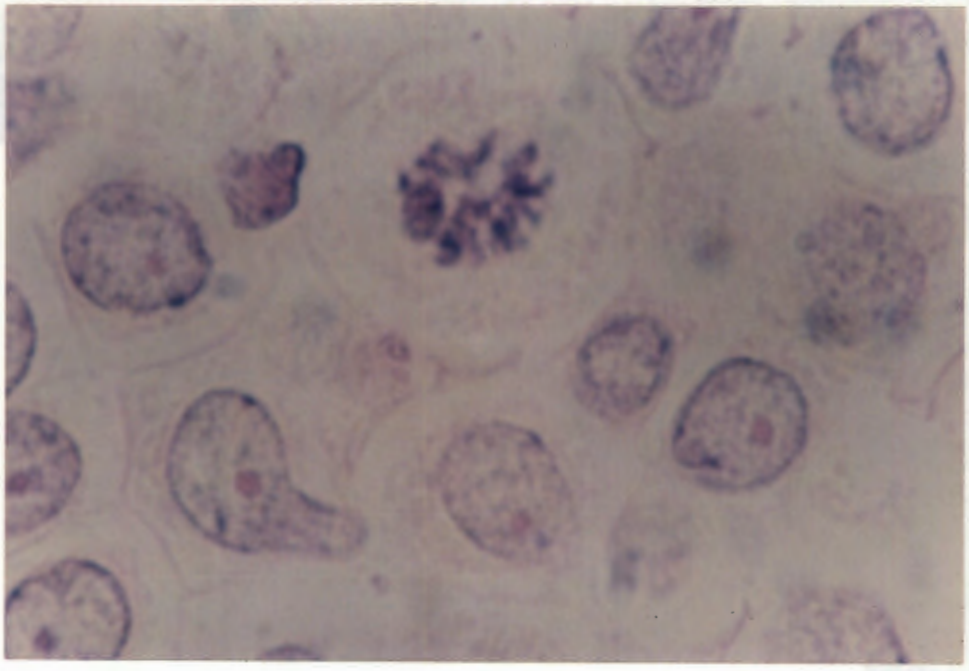
In some reported studies, the aspirate was either inadequate or unsatisfactory in up to 30% of the cases in which a good trephine biopsy was obtained (31). Bone marrow cellularity, extent and homogeneity of the leukaemic infiltrate, as well as residual haemopoietic activity, were more accurately assessed in the sections than in the aspirate (13,28,31,34,35).

Reports have recently been published of the use of myeloid markers in paraffin-embedded specimens, but this still has very limited application, as only a few antibodies can be used (36,18).

Hall *et al* (18) reported demonstrating of lymphoid antigens in decalcified bone marrow trephines, with some limited monoclonal antibodies. However, embedding marrow cores in glycol methacrylate [GMA] and obtaining 2 micron thick sections has improved the diagnostic information available from properly prepared bone marrow biopsy specimens. Aggregates of erythroid precursors, granulocytes at various stages of maturity, monocytes, macrophages, lymphocytes, and megakaryocytes are easily identified (13,34).

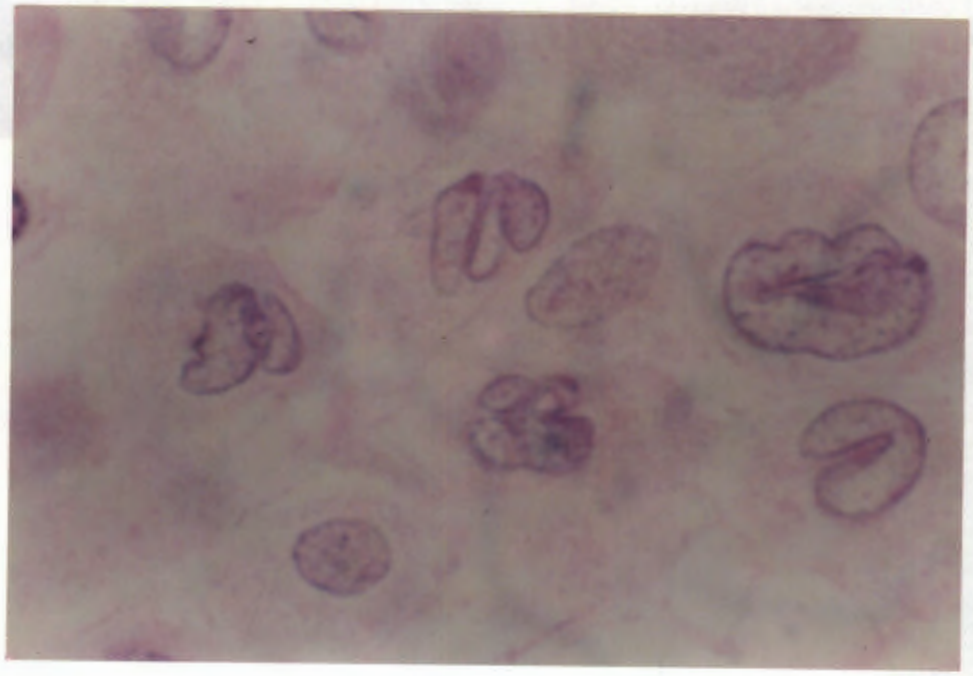
In these sections, myeloblasts show a narrow rim of blue cytoplasm, a relatively round nucleus and a delicate chromatin pattern with one or more small nucleoli.

**Figure 2:** **GMA marrow section from a case of ANLL showing homogenous infiltration of the marrow with blasts. Mitotic figure can be seen. [H&Ex1000] GMA**



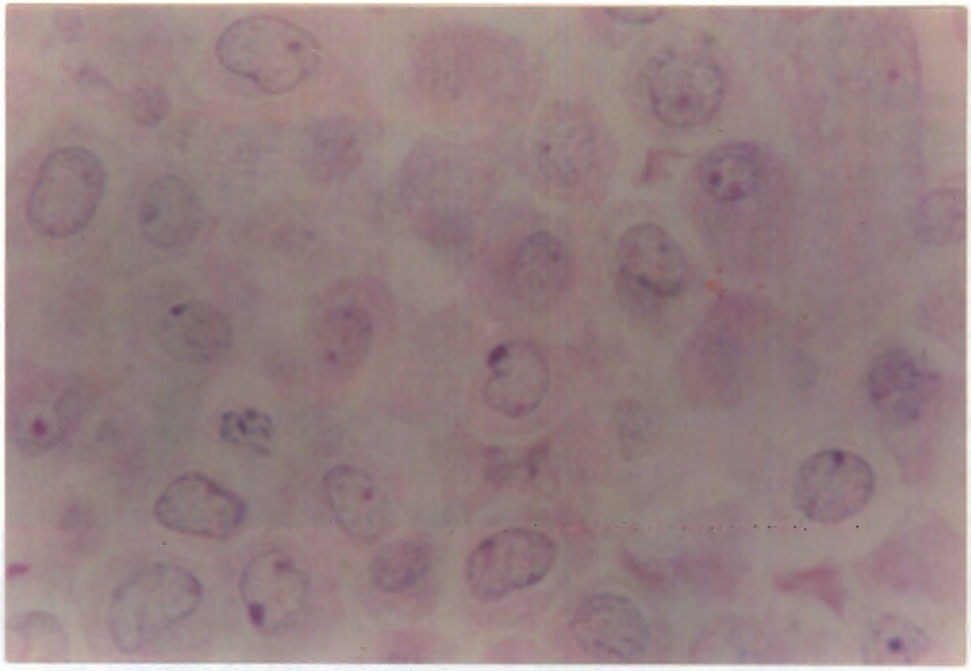
Monoblasts show abundant cytoplasm, a large irregular, convoluted nucleus, and delicate, sometimes lacy chromatin [Fig.3].

**Figure 3:** **GMA marrow section from a case of acute myelomonocytic leukaemia showing easily identifiable monocytic component [cells with irregular, invaginated nuclei] [H&Ex1000] GMA**



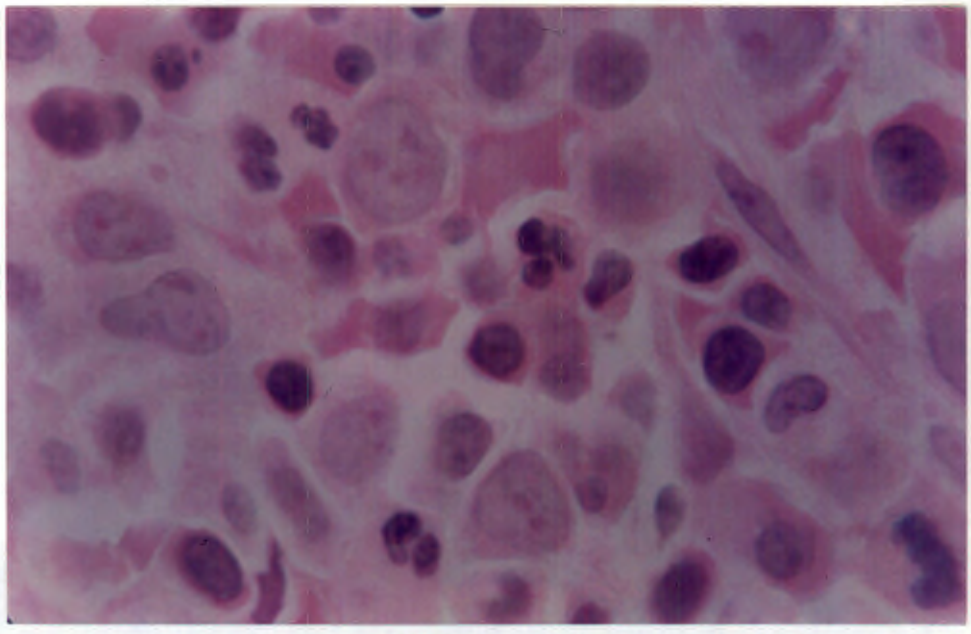
Promyelocytes and myelocytes are easily recognized [Fig.4].

**Figure 4:** **GMA marrow section from a case of acute promyelocytic leukaemia [APL] showing infiltration of the marrow by promyelocytes. [H&Ex400] GMA**



Erythroblasts have a round nucleus, the chromatin has a granular appearance, and the cytoplasm is basophilic [Fig.5].

**Figure 5:** **Prominent features of dyserythropoiesis in MDS - RAEB [multinuclearity, nuclear fragments] [H&Ex600] GMA**



## GLYCOL METHACRYLATE EMBEDDING

Methacrylate resins (methyl and glycol methacrylate) have been used for many years, initially as embedments for ultrastructual studies in electron microscopy and recently as an alternative to paraffin wax embedding for a variety of tissues.
Glycol methacrylate was introduced in 1960 as an embedding medium for electron microscopy by Rosenberg and used for undecalcified bone marrow embedding by Troyer and Nusbickel in 1975 who also noticed that this method resulted in preservation of marrow enzymatic activity (37).

Resin embedding is superior to the paraffin method as it produces less tissue distortion, improves resolution of structural detail and if polymerisation occurs in the cold, there is significant reduction in protein denaturation.
The main disadvantage of this method is the technical problems of achieving uniformity in section production, particularly with methyl methacrylate, as the specimen blocks tend to split and crack. The exothermic polymerization reaction has been another major problem, with the temperatures easily exceeding 100 0C, resulting in distortion of the tissue structure, protein denaturation and production of bubbles in the embedment. The main method of controlling the heat build-up in the tissue blocks has been the use of a water bath to dissipate the rapid rise in temperature.
Alpha-terpinene, a heat moderator, has also been used to prevent excessive temperature rise during polymerisation (26).

The ethylene glycol monester 2 - hydroxyethyl methacrylate may be formed by heating methacrylic acid and ethylene glycol. The produced monomer is a colourless liquid which is hygroscopic, this being a major advantage as this may be used as a dehydrating agent instead of ethanol or acetones.

As with other methacrylates, the GMA is usually mixed with a variety of plasticizers and initiators which improve the cutting properties of the block as well as ensuring reproducible polymerization.

Polyethylene glycol modifies the hardness of the block, while butoxyethanol and N,N - dimethylaniline act as softners and initiators. Induction of the polymerization process takes place by either heat or ultraviolet light provided that benzoyl peroxide is present as a catalyst. (37)

Due to the density of bone specimens, it is necessary to apply vacuum infiltration in order to obtain an even methacrylate penetration in these tissues.

Once polymerized, the blocks may be stored almost indefinitely at room temperature, but cold storage at -20 0C is mandatory if cytochemistry or immunophenotyping is intended.

Conventional microtomes used to cut sections from paraffin blocks are not suitable to cut 2 micron thick sections from GMA blocks, which thus requires the use of specially hardened knives, for example, tungsten knives.

Sections are attached to clean glass surfaces by drying at room temperature without the use of special adhesives.

In recent years, several studies have been published with the use of GMA as an embedding medium for marrow biopsies for the diagnosis and classification of haematological malignancies. (13,28,29,30)

## THE MYELODYSPLASTIC SYNDROMES [MDS]

During the last 40 years haematologists have observed that some of their patients with ANLL had had pre-existing morphological haematological abnormalities which had antedated the diagnosis of leukaemia by months or even years.

These individuals were reported to have had peripheral blood cytopenias. While the marrow was usually hypercellular, the blast count was less than 5%, and there were obvious signs of maturation abnormalities in one or all three haemopoietic lines.
As a result of the introduction of iron stains on bone marrow smears, several cases of sideroblastic anaemia were diagnosed in the 1960's, but refractory anaemia and smouldering leukaemia were not recognized at that time (5).

However, a retrospective study of bone marrows performed then showed that there had been a significant number of megaloblastic and dyserythropoietic marrows which were resistant to both folate and Vit $B_{12}$ therapy, and it was agreed that these would be labelled MDS (5).

At present, the number of newly diagnosed MDS cases is increasing every year. In the United States the incidence of MDS diagnosis is estimated at about one new case for 100 000 persons per year (38).

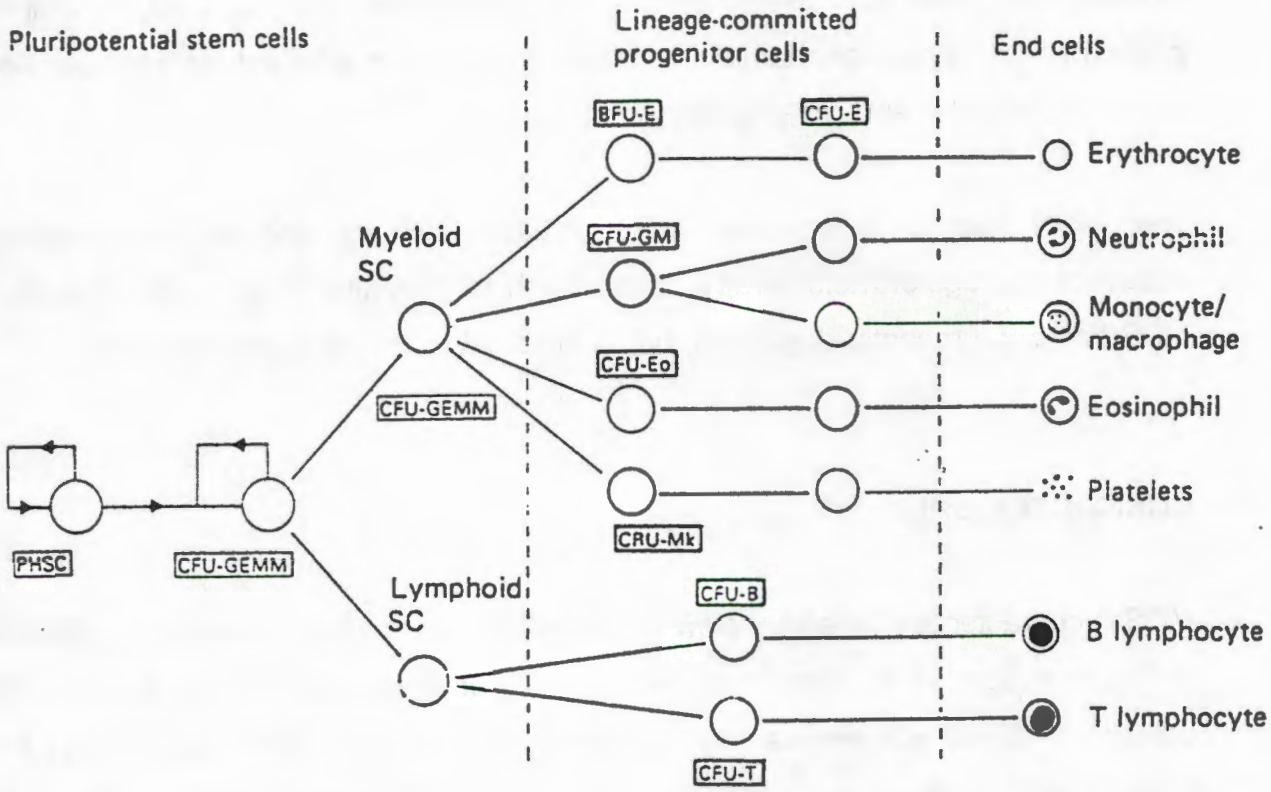
Myelodysplastic conditions may be either congenital or acquired. The latter can be further divided into primary and secondary MDS (5).

**PATHOGENESIS**

The current concept of the MDS's is that they represent the early phase of an acute nonlymphoid (most frequently) leukaemia [ANLL] with a slowly proliferating clone. This clone, which replaces normal haemopoietic cells and is characterized by ineffective and dysplastic haemopoiesis, most commonly results in a hypercellular marrow and peripheral blood cytopenia (1,2,4,5,30,39,40,41,42,43,44). The marrow is hypocellular in only about 10% of all cases (41,44). The defect may be principally manifested in one lineage, but is more commonly bilineal or trilineal. It appears that the abnormal clone has a growth advantage over normal haemopoietic stem cells and will progressively replace them (5,45). These abnormal progeny can be shown by karyotypic analysis to be clonal in origin, particularly in secondary MDS (2,5).

A schematic representation of the developmental pathway for haemopoietic cells is shown in Table 1. MDS and AL represent clonal disorders which are currently thought to arise from mutation in an early stem cell, evolving to ALL or ANLL.

**Table 1:**




**Abbreviations:**

PHSC, pluripotential haemopoietic stem cell; SC, stem cell; CFU-GEMM, colony-forming unit, granulocytic-erythroid-macrophagic-megakaryocytic; BFU-E, burst-forming unit, erythroid; CFU-E, colony forming unit, erythroid; CFU-GM, colony-forming unit, granulocyte-macrophage; CFU-Eo, colony-forming unit, eosinophilic; CFU-Mk, colony-forming unit, megakaryocytic; CFU-B, colony-forming unit, B lineage; CFU-T, colony-forming unit, T lineage (2).

The most common type of MDS is primary MDS in which more than 90% of patients are over 40 years of age with more than 50% being over 70 years (5). According to a recently published study the annual incidence of MDS in children less than 15 years is 3.4/1 000 000 (46).

In secondary MDS patients, there is often a history of cytotoxic drugs use and/or radiotherapy for malignant or non-malignant conditions (2,5). The agents responsible for secondary MDS include alkylating agents - melphalan, chlorambucil, treosulphan, procarbazine, cyclophosphamide, busulphan, razoxane, and the nitrosureas, which produce profound and prolonged cytopenia (2,5).

The risk of developing secondary MDS increases with age and the past duration of exposure to drugs or radiotherapy. It can reach 15-20% after 10 years (5). Congenital MDS has been recently described, but is believed to be extremely rare (47).

## CLINICAL FEATURES

MDS is characterized by a very slow progression, and is often discovered incidentally during investigation for other reasons (2,4). Pallor associated with anaemia is often present. Patients with even a very low haemoglobin concentration [60-80 g/L] have relatively mild symptoms - weakness and exertional dyspnea, which suggests that there has been adaptation over a long period of time. Some individuals may present with infection due to neutropenia, or with bleeding due to thrombocytopenia and platelet dysfunction.

Clinical examination may reveal a palpable spleen, a sign more likely to be found in chronic myelomonocytic leukaemia [CMML] (2). Hepatomegaly occurs only in about 5% of MDS patients (4).

## MORPHOLOGICAL ABNORMALITIES

In 1982 the French-American-British [FAB] Cooperative Group proposed a morphological classification of the myelodysplastic syndromes, (9) and this has since been widely adopted.

The abnormalities of the erythroid, granulocytic and megakaryocytic series are assessed in well-prepared peripheral blood and bone marrow films stained with Romanowsky dyes such as the May-Grunewald-Giemsa stain.

The FAB group proposed that MDS's be defined by the following.

**Dyserythropoiesis:**

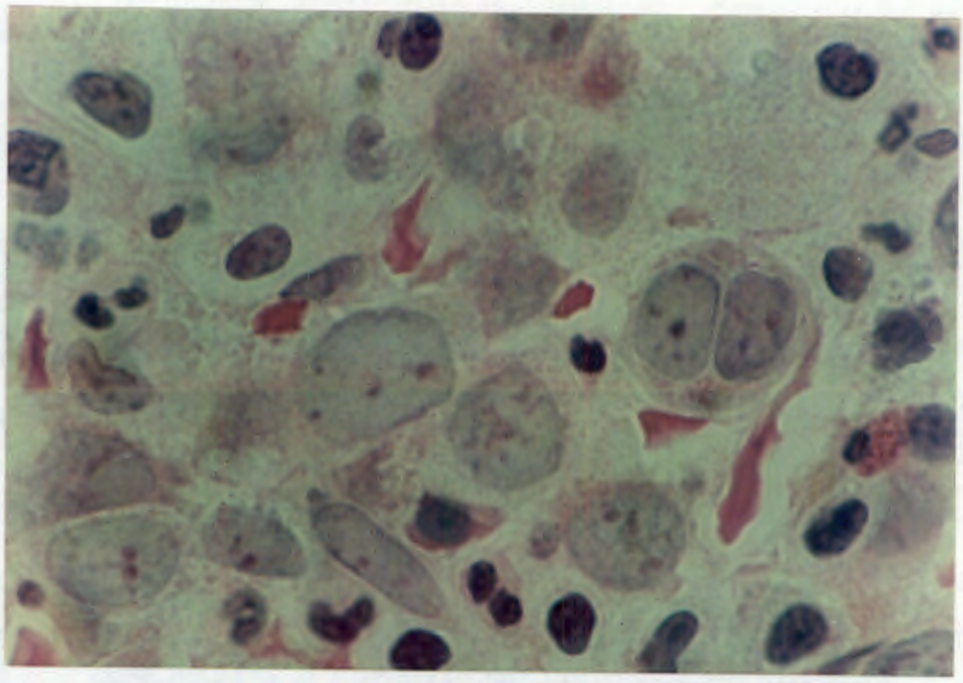
Peripheral blood abnormalities are primarily seen as changes to the red blood cell [RBC] morphology [macrocytes, dimorphism, polychromatic cells, punctate basophilia and, rarely, the presence of nucleated RBC] which is often an indication to look for dyserythropoiesis in the bone marrow. It is believed that these abnormalities are primarily a result of disordered erythropoiesis. Anaemia is an almost universal quantitative abnormality of the erythroid series, and is usually the result of ineffective erythropoiesis. This can be demonstrated by ferrokinetic studies. Quantitative abnormalities of the bone marrow which may reflect extreme defects in RBC production include the presence of more than 15% ring sideroblasts, and the nucleated bone marrow cells having either a greater (more than 60%) or a smaller than usual (less than 5%) proportion of erythroid precursors. Qualitative abnormalities in the bone marrow include the presence of ringed sideroblasts, multinuclearity, nuclear fragments of various sizes, abnormal nuclear shape (e.g. single, double or triple indentations resulting in two or more nuclear lobes of equal or unequal size or an irregular outline) [Fig.5] and abnormal cytoplasmic features (e.g. irregularities in the density of staining which, when extreme, shows as clear unstained areas with ill-defined edges that occupy a variable proportion of the cytoplasm and punctate basophilia) (9).

**Dysgranulopoiesis:**

Morphological abnormalities in granulocytes may be seen in the peripheral blood and the bone marrow. In the peripheral blood the neutrophils may appear as agranular or hypogranular on May-Grunwald-Giemsa stain. In some cases the cytoplasmic

abnormalities are represented by the persistence of basophilia in mature cells. Abnormalities in the nuclear segmentation may be seen as hyposegmentation (Pelger-Huet-like anomaly) [Fig.6] or hypersegmentation with bizarre shapes (9,48).

**Figure 6:** **Presence of ALIP and pseudo Pelgar - Huet forms in MDS - RAEB - t case. [H&E x600] GMA**

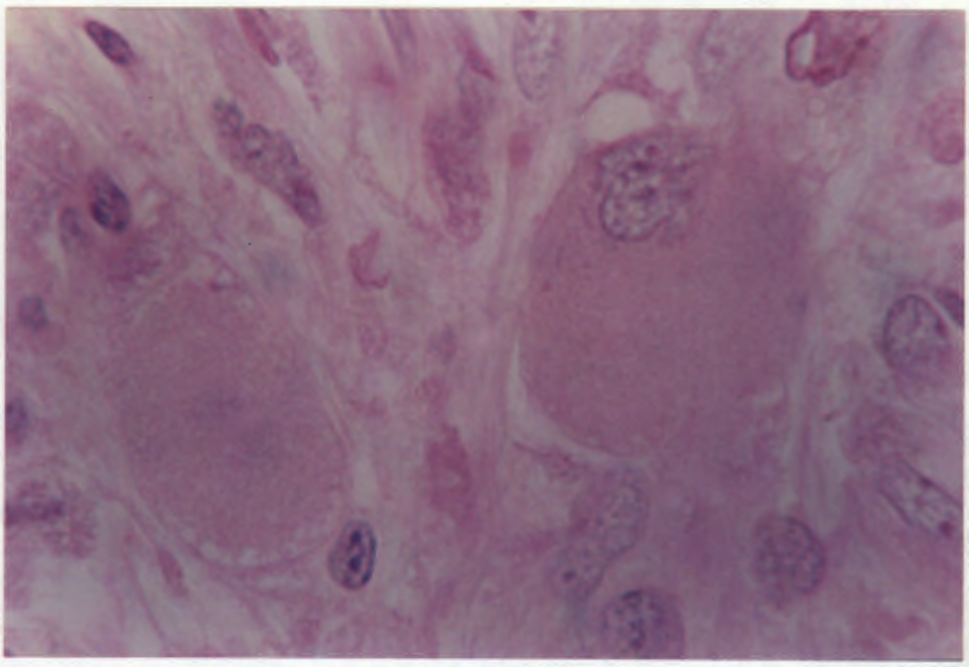


In the bone marrow, abnormal staining of the primary (azurophilic) granules is one of the major abnormalities found in the promyelocytes and myelocytes. In some cases the cells seem to be devoid of granules while in others the primary granules appear larger than normal. In the bone marrow the secondary granules may be absent or reduced in the myelocytes and later forms including mature granulocytes (9). In addition, there may be irregular distribution of the cytoplasmic basophilia, with a dense rim of basophilia in the periphery of the cells, and a lack of it in the perinuclear area. The same nuclear abnormalities of the mature granulocytes that are seen in the peripheral blood are also present in the bone marrow (9).

**Dysmegakaryocytopoiesis:**

Peripheral blood may show the presence of giant platelets, or, rarely, megakaryocyte fragments. Qualitative abnormalities in the marrow are frequently found and sometimes there is a reduction in the number of megakaryocytes as the result of a quantitative defect being also present. Qualitative abnormalities include micromegakaryocytes, [Fig.7] large mononuclear forms and cells with multiple small separated nuclei. Occasionally megakaryocytes with giant and/or abnormal granules are seen. Varying proportions of these abnormal forms and of normal megakaryocytes are found (9,27,49).

**Figure 7:** **Dysplastic megakaryocytes [micromegakaryocytes] in MDS - RAEB. [H&E x600] GMA**



**Blast cells seen in MDS:**

Type I blasts vary from cells which are indistinguishable from myeloblasts to cells of various size which may be unclassifiable. Cytoplasmic granules are always absent and they usually have prominent nucleoli and an uncondensed chromatin pattern. The

nuclear/cytoplasmic ratio of the smaller blasts tends to be higher than that of the larger ones (9).

Type II blasts have a few primary (azurophilic) granules. Otherwise, they resemble type I blasts except that the nuclear/cytoplasmic ratio tends to be lower and the nucleus remains in a central position (9).

If the following characteristics are present, the cells should no longer be considered to be type II blasts and should be classified as promyelocytes - cells with eccentric nucleus and developed Golgi apparatus (seen as a clear zone in the vicinity of the nucleus), with a denser and/or clumped chromatin pattern, and with numerous granules and a low N/C ratio. Deficiency of primary granules or their failure to take up the stain may result in a hypogranular or agranular promyelocyte which can be recognized by the remaining four features (9).

The FAB classification of the myelodysplastic syndromes can be summarized as shown in Table 2.

**Table 2: The FAB morphological classification of the Myelodysplastic Syndromes**

| Category | Blood | Bone marrow |
|---|---|---|
| 1. Refractory anaemia [RA] | <1% blasts | <5% blasts<br><15% RS [ring sideroblasts] |
| 2. RA with ring sideroblasts [RARS] | <1% blasts | <5% blasts<br>>15% RS |
| 3. RA with excess of blasts [RAEB] | <5% blasts | 5-20% blasts |
| 4. RAEB in transformation [RAEB-t] | >5% blasts | 21-29% blasts |
| 5. Chronic myelomonocytic leukaemia [CMML] | <5% blasts<br>> 1 x $10^9$ monocytes | <20% blasts. Excess of monocyte precursors |

The diagnosis is RAEB-t if Auer rods are present in bone marrow or peripheral blood blasts (9).

**CYTOGENETIC ABNORMALITIES**

Karyotypic abnormalities in the haemopoietic cells have been reported in about half the MDS patients (2,4,39,50,51,52,53,54). Interestingly, the chromosomes most frequently involved carry genes important for haemopoiesis, e.g. chromosomes 5 and 7, which contain genes for haemopoietic growth factors and its receptors, and for erythropoietin respectively.

Recently, several haemopoietic growth factors [GM-CSF, M-CSF, Il-3,Il-4, Il-5] as well as the c-fms proto-oncogene, were located on the distal arm of chromosome 5 (6,55). The most common cytogenetic anomalies in MDS involve chromosome 8, [+8 or trisomy 8], chromosome 7, [-7 or monosomy 7] and chromosome 5 [-5 and 5q- or interstitial deletion of part of the long arm of chromosome 5 (2,5,6,50).

Chromosome 8 carries the c-myc oncogene, and the portion of chromosome 5 which is frequently lost in MDS and ANLL corresponds to the c-fms oncogene. Amplification of the c-myc oncogene has been reported in blastic crisis of chronic myeloid leukaemia [CML] and in ANLL (6,53). On the normal chromosome, c-myc oncogene is transcriptionally silent. The relocation of c-myc leads to the persistent activation of this gene and may play a key role in cell transformation (54).

Involvement of chromosomes 5 and 7 is prominent in secondary MDS but, in contrast to primary MDS, chromosome 8 remains unaffected (5). The presence of the -7 chromosomal abnormality predicts a higher incidence of transformation to overt ANLL than do abnormalities of chromosome 5 (6).

Chromosome patterns which are not seen in MDS are t[8;21] and t[15;17].

Karyotype anomalies are useful confirmatory evidence for an MDS clone, and can confirm a doubtful diagnosis in some patients.

Chromosomal abnormalities associated with MDS are listed in Table 3.

**Table 3:**

| Category | Cytogenetic abnormalities |
|---|---|
| 1. Refractory anaemia. [RA] | 5q-, +8, -7 |
| 2. RA with ring sideroblasts. [RARS] | 5q-, +8, -7<br>20q-, 11q- |
| 3. RA with excess of blasts. [RAEB] | 5q-, +8, -7, -5 |
| 4. RAEB in transformation. [RAEB-t] | 5q-, +8, -7, -5 |
| 5. Chronic myelomonocytic leukaemia. [CMML] | -7, +8, 12p- |

**FUNCTIONAL ABNORMALITIES**

Various defects in red cells, granulocytes and platelets in MDS have been described (2,5,6,44) They include altered function of glycolytic enzymes in red cells [pyruvate kinase deficiency], increased concentration of Hb-F [80% of patients with MDS], and altered expression of blood group antigens.

Other enzyme activities like hexokinase and glucose-6-phosphate dehydrogenase frequently exhibit increased activity (5).

Granulocyte abnormalities include defective adhesion, orientation, aggregation, chemotaxis, phagocytosis, degranulation, decreased myeloperoxidase content, release and killing capacity against bacteria and yeasts (2,5,44,56).

However, granulocyte function in patients with RARS did not differ from normal controls, implying that these individuals may form a separate entity (5). Lymphocytes were found to have decreased DNA-repair potential, while alterations in natural killer cell activity were also reported (56).

Platelet abnormalities include defective aggregation, adhesion to glass, and reduced thromboxane $A_2$ activity (2). The platelet count remains the most important factor in

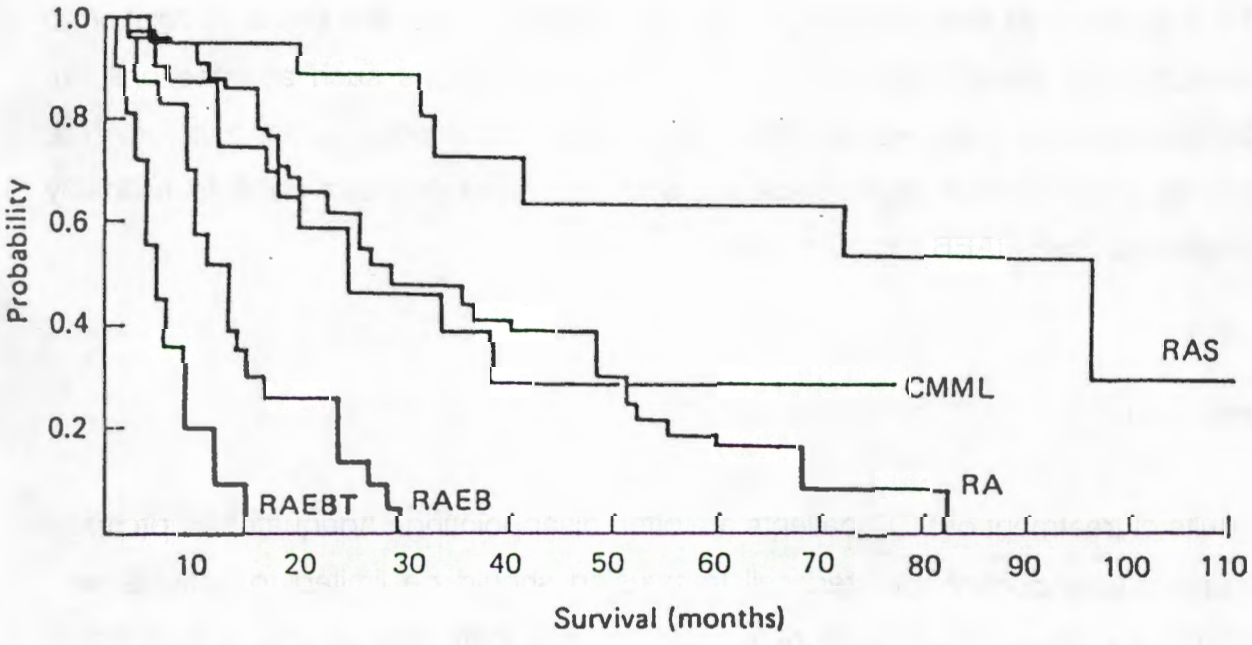
the prediction of a bleeding tendency, and there have been only a few reports of haemorrhage and thrombosis due to abnormal platelet function (2,4,5).

**PROGNOSIS**

Problems related to cytopenia limit survival in patients with MDS. Anaemia and transfusion-related problems, agranulocytosis and thrombocytopenia are more frequent causes of death than is a transformation to ANLL.

The median duration of survival in large studies varies according to the morphologic subclass, being 76 months for RARS, 32 months for RA, 10,5 months for RAEB, and only 5 months for RAEB-t (2). The median survival for CMML is 38 months (2) [see Table 4].

**Table 4:**




(2)(Mufti *et al*)

The bone marrow blast cell count is the single most important determinant of the prognosis - the duration of survival being markedly worse when the blast-cell count is greater than 5%, (32,57,58,59) but, in a recently published study, White *et al* (51) showed that karyotype status is also an important prognostic indicator, not only in relation to survival, but also to the risk of leukaemic transformation.

Secondary MDS carries a very poor prognosis with a disappointing response to treatment and short survival times because of its faster evolution to acute leukaemia (58).

De Wolf-Peeters *et al* (30) reported that the abnormal localization of immature cells [ALIP] predisposes MDS patients to a progression to overt AML.

ALIP is defined as being the presence of abnormally located myelopoiesis, clustering centrally in the marrow with a shift to more immature forms - namely myeloblasts and promyelocytes (1)[Fig.6].

The absence of ALIP seems to carry a better prognosis, but this bears no relation to morbidity and mortality as a result of other complications such as infections (6). Cosson *et al* (60) stressed that there was a significantly worse prognosis for ANLL patients with trilineage myelodysplasia, and that many of these patients probably progressed from RAEB-t to overt ANLL.

**TREATMENT**

Results of treatment of MDS patients are often disappointing. Adequate symptomatic treatment is important, but red cell transfusion should be limited to patients with symptoms of anaemia, as some of them, in the long term, may develop iron overload as a result of frequent blood transfusions.

Prompt treatment with the appropriate antibiotics is warranted in neutropenic patients, as they are susceptible to infections that can be lethal (2,6). Platelet transfusions are given when bleeding is a problem, or in the case of extreme thrombocytopenia. Patients with splenomegaly may develop hypersplenism requiring splenectomy (2). RARS and RA both have a relatively good prognosis in the short term and are managed conservatively with supportive therapy - blood and platelet transfusions (2,5,39,43,61,62,63).

At present therapeutic regimens for the high-grade MDS are under investigation (2,4,5,39,43,61). These involve the use of putative stem-cell differentiating agents, such as cytosine arabinoside [ara-C] at low dosage, generally with 10-20% of individuals achieving complete remission.

However, the durability of these responses was usually short and no improvement in survival was noted. In younger patients, marrow ablative treatment followed by allogenic bone marrow transplantation has been reported in recent studies to achieve 45% disease-free survival for three years, with 23% having a probability of relapse (61).

This treatment is usually restricted to patients below the age of 40 who have a histocompatible bone marrow donor. However, the majority of MDS patients are above this age, and therefore are not appropriate candidates for bone marrow transplantation (2,5).

Recently, the use of haemopoietic growth factors - granulocyte colony-stimulating factor, [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF], and interleukin-3 [Il-3] - have been shown to improve the neutrophil counts and marrow morphology in a high proportion of patients, and for G-and GM-CSF these responses, although initially present, were short lived. In some subjects a decrease in red cell transfusion requirements occurred, blood leucocyte counts increased significantly, whereas platelet counts remained unchanged (6). Thus, haemopoietic growth factors provide encouraging alternatives to chemotherapy or bone marrow transplantation for

elderly patients, as they improve marrow recovery, although the effect is limited to the period of administration of the agent.

### EVOLUTION OF MDS

Patients with MDS may die of marrow failure as a direct consequence of disease, or following transformation to acute leukaemia, which may develop within a short time or evolve in a stepwise fashion over many weeks, month or even years.

Myelodysplastic syndromes may also progress into other usually less favourable categories. Thus RA and RARS may evolve into either CMML or RAEB, both of which may in turn progress to RAEB-t (6,59). Patients with RAEB and RAEB-t show a high incidence of evolution to ANLL - 42% and 59% respectively - in contrast to RA and RARS cases with 12,5% and 11,5% respectively (60). The acute leukaemia which occurs in MDS is almost always ANLL, but rare cases of ALL and of bilineage / biphenotypic leukaemia have been reported (6). Since MDS is a disease of the elderly, a significant proportion of patients with MDS succumb to other diseases.

## ACUTE LEUKAEMIA [AL]

Acute leukaemia [AL] is a malignant proliferation of immature haemopoietic cells in the bone marrow which arises as a result of somatic mutation of a single haemopoietic stem cell (2,6). Leukaemia is probably a multiple-step process that requires the altered expression of several interrelated genes in the same cells. Myeloid leukaemia arises in multipotent stem cells that are capable of differentiating into cells of myeloid, erythroid, monocytic, and megakaryocytic lineages (2,6). The leukaemic blast cells accumulate in the marrow and suppress the proliferation and differentiation of normal haemopoietic cells, leading to diminished production of erythrocytes, granulocytes and platelets.

Acute leukaemias are divided into two major groups - acute non-lymphoblastic leukaemia [ANLL], and acute lymphoblastic leukaemia [ALL].

In addition, hybrid acute leukaemias have recently been recognized in which leukaemic cells express markers of two lineages (4,64,65,66,67). When more than 10% of malignant cells in a hybrid leukaemia demonstrate both lymphoid and myeloid features, the leukaemia is termed biphenotypic. However, if the leukaemic cells are heterogeneous with single cells displaying either lymphoid or myeloid features but not both, the leukaemia is termed bilineal or biclonal.

## AETIOLOGY

The cause of acute leukaemia in humans is not known. Several factors may play a role in the induction of certain types of leukaemia, including the following:

1. **Environmental agents:**

   - **ionizing radiation:**
     The leukaemogenic potential of ionizing radiation has been known for years. It increases with the amount, quality and time of exposure (2,4,68).

   - **viruses [retroviruses]:**
     Retroviruses carry oncogenes that are caused by the transduction of genes [proto-oncogenes] of vertebrate cells. The growth of cells is critically regulated by proto-oncogenes: others code for growth factors, such as platelet derived growth factor, encoded by c-sis; others code for the receptors of growth factors, such as the receptor for macrophage colony- stimulating factor, encoded by c-fms; some code for transducers within the cell that help ramify a cell surface signal, such as ras oncogenes (2,4,6,9,).

ANLL appears to exhibit the highest frequency of ras oncogene activation of any human tumour so far studied (6,69). To date, oncogene analysis does not play a role in the diagnosis of acute leukaemia. However, since leukaemogenesis is a multistep process in which proto-oncogenes are activated by point mutation, truncations, chromosomal translocations, and/or over- or under-expression, oncogenic expression at numerous molecular levels may become more important in diagnosis.

- **chemical carcinogens [benzene]:**
  The incidence of acute leukaemia in workers exposed to benzene is two to ten times greater than in the general population ((2,4,70,71,72).

- **alkylating agents [melphalan, busulphan, chlorambucil]:**
  The combination of irradiation and alkylating agents has resulted in a higher incidence of "secondary" leukaemias with the time between the original malignant diagnosis and the development of bone marrow haematological malignancy being between 3 and 5 years (2,4,5,73,74).

Patients developing ANLL include those with Hodgkin's and non-Hodgkin's lymphoma, multiple myeloma, hairy cell leukaemia, and various solid tumours. These leukaemias have also been shown to be associated with a high incidence of chromosomal abnormalities involving chromosomes 5 and 7 (2).

2. **Genetic condition:**

Individuals with hereditary disorders such as Down's syndrome, Bloom's syndrome, Fanconi's anaemia, ataxia telangiectasia, and congenital agammalobulinemia are prone to develop acute leukaemia. Down's syndrome is associated with stable trisomy 21, whereas Bloom's syndrome, Fanconi's anaemia, and ataxia telangiectasia are autosomal recessive disorders which are

associated with multiple chromosomal breaks, aberration, and endoreduplication in cultured cells (2,4,6).

3. **Acquired diseases:**

Pre-existing conditions such as myelodysplastic syndromes, aplastic anaemia, multiple myeloma, and paroxysmal nocturnal haemoglobinuria (2,4).

**CLINICAL PRESENTATION**

ANLL has a low incidence in childhood, less than 1 case/100 000/year, and incidence rises with age from 1/100 000/year in the fourth decade to approximately 10/100 000/year in those over 70 (38).

Patients present with nonspecific systemic manifestations, such as fatigue,weight loss, fever or bruising. As a result of marrow infiltration, the patient develops anaemia, leucopenia and thrombocytopenia which lead to prostration, severe infections and bleeding.

Thrombocytopenic patients present with a history of easy bruising, petechiae, epistaxis, gingival bleeding and prolonged bleeding after injuries. Bleeding may occur from any site, and haemorrhages potentially fatal are intracranial, gastrointestinal and pulmonary. Less common symptoms of acute leukaemia include retinal haemorrhages and the development of extranodal masses in the subcutaneous tissues, orbits, breasts, or testes (4).

Some patients develop persistent or recurrent skeletal pain or tenderness, such as sternal pain or swelling of the large joints (2,4). Leukaemic patients may have organ infiltration (eg. liver, lymph nodes or brain), this being more common in ALL. Infiltration of the gingivae and skin is fairly common in myelomonocytic and monocytic ANLL. Meningeal leukaemia occurs in ANLL in only about 5% of adults as compared to 70% of those with ALL.

The most serious and life-threatening complication of leukaemia is infection which develops as a result of the breakdown of normal defence mechanisms. Natural barriers to infection, adequate inflammatory responses, and cellular and humoral immunity are all disrupted by leukaemia. The risk of infection is particularly high when the granulocyte count decreases to below 0,5 $x10^9$ /L.

Pyogenic infections of the skin are most common, with major infections such as sinusitis, pneumonia, pyelonephritis and meningitis being rare as presenting features.

Leukaemic therapy itself injures the gastrointestinal mucosa, creating a portal of entry for microorganisms. Venipunctures, marrow aspirations and biopsies and bladder catheterizations result in local injury which may lead to serious infection (4).

**DIAGNOSIS**

At present the differentiation and classification of AL not only requires simple morphological and cytochemical evaluation, but, in some cases, it is also necessary to use monoclonal antibodies, electron microscopy or molecular biological techniques such as gene rearrangement studies (2, 4, 6, 7, 8, 10, 11, 12, 20, 36, 38, 64, 65, 66, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89 ,90, 91, 92, 94).

In 1976 the FAB Cooperative Group published proposals for the morphological classification of the acute leukaemias, (8) which have been widely adopted and used. These proposals were revised by the FAB Cooperative Group in 1985, (11) and criteria for M7 were added (10). In 1991 criteria for M0 (7) were added.

The presence of more than 30% of type I or type II blasts in the marrow is diagnostic of acute leukaemia (8,11).

**The FAB morphological classification of ANLL** (7,8,10,11,12)

1. M0 Undifferentiated myeloblastic
2. M1 Myeloblastic without maturation
3. M2 Myeloblastic with maturation
4. M3 Promyelocytic
   M3V Promyelocytic - variant
5. M4 Myelomonocytic, with both granulocytic and monocytic differentiation
   M4E Myelomonocytic, with eosinophilia
6. M5 Monocytic
   M5a - Monoblastic
   M5b - Monocytic
7. M6 Erythroleukaemia [with >50% erythroblasts and >30% of nonerythroid cells being blasts]
8. M7 Megakaryoblastic

M0 - cannot be diagnosed on morphological grounds alone as the blasts are large and agranular. They should be identified by the following features: negative myeloperoxidase and Sudan Black B reaction [or positivity in less than 3% of blasts], negative B and T lineage markers, and expression of myeloid antigens recognized by at least one monoclonal antibody, either CD 13 or CD 33.

Other myeloid markers are often also positive, and these include CD 11b and the enzyme myeloperoxidase [MPO] demonstrated by immunocytochemistry and/or electron microscopy analysis (7).

M1 - the sum of blast cell types I and II must be 90% or more of all the nonerythroid cells with at least 3% of the blasts being myeloperoxidase or Sudan Black B positive and a varying proportion of blasts containing at least a few azurophilic granules, Auer rods or both (8,11).

M2 - the sum of blast cell type I and II is between 30% and 89% of the nonerythroid cells. Monocytic cells make up less than 20% of the total and maturation at or beyond the promyelocyte stage is present in more than 10% of the bone marrow cells.

The leukaemic cells are often nucleolated and have varying amounts of cytoplasm, usually with many azurophilic granules. Cells containing Auer rods, almost always single, are common (8,11).

M3 - the great majority of cells are abnormal promyelocytes, with a characteristic pattern of granulation, and with bundles of Auer rods almost invariably being present in some of the cells in the peripheral blood and marrow. Promyelocytes nuclei vary greatly in size and shape and are often reniform or bilobed (8,11,12).

M3 variant - is characterized by a cell with a reniform, bilobed, multilobed or convoluted nucleus and with either sparse fine granules or agranular cytoplasm.

A variable proportion of cells may have multiple Auer rods, fine dust - like granules, or large oval or elliptiform cytoplasmic inclusions with the same staining characteristic as primary granules. Typical hypergranular promyelocytes constitute a small minority of the leukaemic cells in peripheral blood. They are usually more numerous in the bone marrow. The white count is usually considerably higher in M3 variant than in M3 (38).

M4 - the diagnosis of M4, and its separation from M2 and M5, require assessment of both the peripheral blood and the bone marrow films. In bone marrow, the blast cells account for more than 30% of the nonerythroid cells. The sum of myeloblasts, promyelocytes, myelocytes, and later granulocytes is 30% or more but less than 80% of the

nonerythroid cells. More than 20% of the nonerythroid cells are cells of the monocytic lineage at different stages of maturation; usually they are promonocytes and monocytes. When the monocytic cells exceed 80%, the diagnosis is M5 (monocytic leukaemia.)

When the bone marrow findings are as above, and the peripheral blood monocyte count (monoblasts, promonocytes, monocytes) is $5 \times 10^9$ /L or more, the diagnosis is M4.

If the monocyte count is less than $5 \times 10^9$ /L, a diagnosis of M4 can still be made if the bone marrow findings are as described above, and the presence of a significant monocytic component has been confirmed by ancillary laboratory tests, such as serum lysozyme estimations, or by cytochemical methods that incorporate either a double esterase reaction, specific chloroacetate esterase and non-specific alpha-naphthyl acetate esterase, or other esterase stains that identify monocytes, such as naphthol ASD acetate, with and without incubation with sodium fluoride.

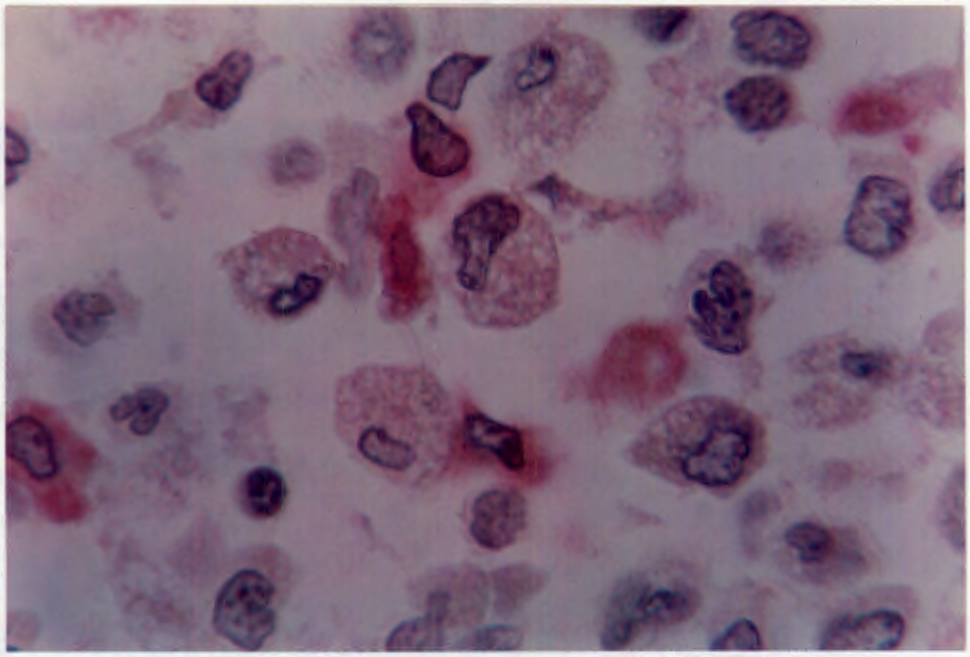
The diagnosis of M4 is established if more than 20% of the bone marrow precursors are monocytes, as shown by cytochemical reactions, or if the lysozyme concentrations exceed three times the normal values in serum (11.54 μg/mL), urine (2.5 μg/mL), or the reference values in individual laboratories. If the bone marrow resembles that of cases of M2, a diagnosis of M4 is still possible if the peripheral blood monocyte count is $5 \times 10^9$ /L or more and one of the above tests provides evidence for an increased monocytic component in the bone marrow (8,11).

M4 - with eosinophilia [M4E]

In a small percentage of cases of M4, eosinophils are present in the bone marrow; they usually amount to 5% or more of nonerythroid cells. These eosinophils are abnormal, and some have large basophilic (immature) granules, in addition to the characteristic specific eosinophilic

granules, and may have a single unsegmented nucleus. In contrast to normal eosinophils, these cells have cytochemical reactions to chloroacetate esterase [Fig. 8] and periodic acid Schiff that are distinctly positive.

**Figure 8:** **Chloroacetate esterase positivity in coarse granules of abnormal eosinophils in a case of M4E. [x600] GMA**



The FAB Cooperative Group proposed that such cases be designated as "M4 with eosinophilia". Although cells with these features can rarely be identified in cases of M2, the cases with M2 and bone marrow eosinophilia generally lack the abnormal eosinophil granules seen in M4 with eosinophilia (8,11).

M5 - the diagnosis of M5 is based on the appearance of the bone marrow. One criterion suffices: if 80% or more of all the nonerythroid cells in the bone marrow are monoblasts, promonocytes, or monocytes. The definitions of the two subtypes M5a and M5b are modified as follows: for M5a, 80% or more of all the monocytic cells are monoblasts; and for

M5b, less than 80% of all the monocytic cells are monoblasts, the remainder being promonocytes and monocytes (8,11).

M6 - to avoid confusing M6 with other megaloblastic and dyserythropoeitic states, the FAB group specified that more than 30% of all the bone marrow cells had to be myeloblasts or promyelocytes. Using this criterion, the FAB group have since recognized that in some cases of M6, in which a high proportion of the erythroblasts were bizarre cells, the erythroid component was so large that it was impossible to diagnose M6 from the original FAB definition as the blast cells accounted for less than 30% of all nucleated cells of the bone marrow.

They revised the criteria for the diagnosis of M6 by requiring that 30% or more of the remaining nonerythroid cells be type I or type II blast cells. The proposed criteria for the diagnosis of M6 are thus different from those used for the other subtypes of acute myeloid leukaemia (M1-M5). Subtype M6 can now be diagnosed when less than 30% of all nucleated cells of the bone marrow are blast cells; some of these cases would previously have been classified as refractory anaemia with excess blast cells (RAEB) or with excess blast cells in transformation (RAEB-t) with a major erythroid component. When 50% or more of all nucleated cells are erythroblasts, the diagnosis becomes myelodysplastic syndrome if less than 30% of the nonerythroid cells are blast cells (8,11).

M7 - leukaemic megakaryoblasts are frequently highly pleomorphic (10). Prominent and multiple nucleoli and cytoplasmic basophilia have been noted. There is some heterogeneity in cell size with approximately 20-30% of the blasts being two or three times larger than normal lymphocytes. At times, cells in either the bone marrow or peripheral blood may have cytoplasmic blebs. The nature of megakaryoblasts may be suggested by the pattern of cytoplasmic reactions - they do not stain with Sudan Black B or peroxidase, are almost always negative for alpha-

naphthyl butyrate esterase, [ANBE] and are frequently PAS and acid phosphatase positive with a localized pattern.

The ultrastructural examination may show that a positive peroxidase activity in megakaryoblasts is localized exclusively on the nuclear membrane and the endoplasmic reticulum, whereas in myeloblasts the reaction occurs in the Golgi area and cytoplasmic granules.

The use of immunological techniques may demonstrate positive reactions for factor VIII-related antigen, platelet factor 4, platelet derived growth factor and platelet membrane glycoproteins.

Myelofibrosis or increased bone marrow reticulin are prominent aspects in most patients with M7 (10).

**CYTOCHEMISTRY**

The rationale for the use of enzyme and immunologic markers in the classification of malignancies is that during differentiation each cell line normally synthesizes particular chemical substances that enable the cells to perform their unique functional role when they mature and are fully differentiated (94,95,96). Many of these substances are unique to one cell line, and may appear at a specific stage of maturation. If leukaemic cells can be shown to contain such substances, they can be considered by analogy with normal cells to be of a particular cell lineage.

**Four types of cytochemical stains were found to be essential for the diagnosis and precise classification of ANLL:**

1. Myeloperoxidase [MPO] - which is formed very early in normal cells committed to nonlymphoid maturation and has never been reported in lymphoid cells (22). Myeloperoxidase activity is found in primary granules of the granulocytic series

and lysosomal granules of the monocytic series. It is found in cell lines developing into neutrophils, eosinophils, basophils, and monocytes (95,96).

Myeloblasts may or may not stain with myeloperoxidase at the light microscopic level, but they may stain the perinuclear cisternae and endoplasmic reticulum prior to packaging into granules at the electron microscopy level.

2. The monocyte has ectoenzymes that readily hydrolyses alpha-naphthyl acetate esterase [ANAE], alpha-naphthyl butyrate esterase [ANBE], or the naphthol-AS acetate esterase [NASA] at acid pH, which gives a characteristically strong diffuse reaction in monocytic leukaemias, M4 or M5. All these esterase reactions are sensitive to inhibition by sodium fluoride (22,96). Granulocytes and lymphocytes are usually negative for ANAE and ANBE, but helper T-cells show dense localized dotlike positivity with ANAE.

   The monocytic leukaemias [M5] are defined by the presence of diffuse ANBE and the absence of naphthol-AS chloracetate esterase [CAE]; peroxidase may or may not be present (2,6,22,70).

3. Naphthol-AS chloracetate esterase is reported to be useful in the diagnosis of myeloblastic leukaemias and, in cases in which peroxidase is negative, can be used on its own for diagnosis. Chloroacetate esterase is positive in myeloid haemopoietic cells, and has also been found to be positive in abnormal eosinophils of M4E and mast cells (22,32,64,96).

   The myeloid leukaemias are thus defined by the presence of peroxidase [at light microscopy or ultrastructural level] and/or CAE and the absence of ANBE (2,6,22,70). Leukaemias with cells containing both ANBE and CAE should be designated as myelomonocytic (8,11).

4. Sudan Black B [SBB] - stains a variety of lipids, including neutral fats, phospholipids, and sterols, but may also stain some cellular elements that are not lipids.

   Lymphocytes and lymphoblasts may rarely stain with Sudan Black B, while granulocytic precursors and immature monocytes show characteristic [strong localised and dense cytoplasmic] staining patterns.

Myeloperoxidase, SBB and chloroacetate esterase have been reported to be much more sensitive for the detection of Auer rods than are the Romanowsky stains (92). Another advantage of these stains is that the agranular mature neutrophils in MDS or M2 cases can be recognized (23,34).

Acid phosphatase shows a distinct localized pattern in the Golgi zone in T-ALL, and also in M6 and M7 blasts (2,6,38,70).

Cytochemistry still remains the cheapest and quickest way of differentiating some types of the AL, but recently developed monoclonal antibodies for myeloid, monocytic, lymphoid, megakaryocytic and erythroid antigens have made the use of cytochemistry less essential.

**IMMUNOLOGICAL CLASSIFICATION**

Immunological techniques are used to identify cytoplasmic and surface membrane antigens of leukaemic cells. Surface markers have been analyzed by various methods -immunofluorescence microscopy, immunocytochemistry and flow cytometry. The first two techniques have the advantage of direct cell analysis, but disadvantages include slow, tedious analysis. Flow cytometry has the advantages of a faster analysis, objectivity, reproducibility, and the ability to estimate cell size and granularity (6).

The expression of monoclonal antibody-defined myeloid antigen coincides with the pathways of normal haematopoietic differentiation within the myeloid lineage. [see Table 5] Immunological markers are important in the diagnosis of ANLL for many reasons. They allow some ANLL, which give negative reactions with SBB, MPO, and non-specific esterase, to be identified as myeloid and inappropriate antigen expression to be identified as bilineage and biphenotypic leukaemia.

Immunophenotyping is particularly important in the diagnosis of MO, M6 and M7, and when it is used to supplement the other subtypes there is an improvement in diagnostic accuracy.

Approximately 3% of ANLL are negative in cytochemical tests, but show positivity with at least one myeloid monoclonal antibody CD13 or CD33 and the enzyme MPO demonstrated by immunocytochemstry and/or electron microscopy; these have been designated as MO (7). Monoclonal antibodies CDw41 and CDw42 have been used in flow cytometry and immunoalkaline phosphatase techniques to establish a diagnosis in acute megakaryocytic leukaemia (2,6,10,70).

Numerous monoclonal antibodies [McAb] are now available which help to identify specific types of ANLL (2, 7, 10, 11, 20, 22, 35, 64, 65, 76, 78, 79, 80, 81, 82, 87, 88, 89, 90, 93, 97, 98, 99).

In general, these reagents do not react with lymphoblasts and have variable expression in the various type of ANLL. Some, such as CD 13, CD 33 and anti-MPO, are broad panmyelomonocytic markers that can be used to screen ANLL cases as they mark early myeloid precursors (83,91). Anti-MPO monoclonal antibodies were reported to be positive in up to 94% of ANLL, with 86% and 84% of CD 13 and CD 33 positivity respectively (88). Also, monoclonal antibodies to glycophorin, the transferrin receptor [OKT-9] and CD 45, have been useful in difficult cases of M6 [see Table 5].

**Table 5: Cell markers in ANLL (2)**

| McAb | M0 | M1 | M2/M3 | M4/M5 | M6 | M7 |
|---|---|---|---|---|---|---|
| CD34 | + | + | +/- | +/- | - | +/- |
| CD13 | + | + | + | + | + | + |
| CD33 | +/- | + | + | + | +/- | + |
| CD11b | - | - | + | +/- | - | - |
| CD14 | - | - | - | + | - | - |
| Glycophorin | - | - | - | - | + | - |
| CDw41/41 | - | - | - | - | - | + |
| TdT | -/+ | -/+ | - | - | - | - |

**CYTOGENETIC ABNORMALITIES**

Mitotic disturbances in tumour cells were recognized by pathologists as early as the late 19th century. Identification in 1960 by Nowell and Hungerford of the Philadelphia chromosome in leukaemic cells from patients with chronic myelogenous leukaemia was a major breakthrough in the detection of chromosomal abnormalities.

A new period began in the 1970's when banding techniques were developed that allowed better detection and definition of specific structural abnormalities not previously recognized. Finally, the current era, which began in the 1980's, has been characterized by considerable improvements in the quality of chromosome preparations available for meticulous analysis. More recently, techniques using cell synchronization and high resolution banding methods have facilitated the precise identification of non random chromosomal abnormalities in a variety of malignancies. As a result of these improvements such derangements have been reported in at least 50% of patients with ANLL, although recent studies suggest that they may be as high as 80% (2).

Both chromosome gains and losses may be seen in ANLL. With the exception of chromosome 16, which has never been observed as a gain, and chromosome 1,

which was never lost, all the other chromosomes have contributed to numerical changes. The gain of chromosome 8, the most frequent abnormality seen in ANLL, results in trisomy 8 and has been observed in several FAB subgroups. The t[8;21] translocation occurs predominantly in the FAB M2 subtype but has been observed in the M4 and M1 subtypes.

The t[15;17] translocation found in the FAB M3 is most specific in that it occurs only in acute promyelocytic leukaemia. Recently, the molecular features underlying the t[15;17] translocation have been clarified.

The breakpoint on chromosome 17 has been localized within the retinoid acid receptor alpha [RAR alpha] locus while that on chromosome 15 has been localized within a gene named promyelocytic [PML]. It is believed that the PML-RAR-alpha fusion product may generate an RAR alpha mutant that contributes to acute promyelocytic leukaemogenesis through interference with promyelocytic differentiation (6).

The inv[16] and t[16;16] abnormalities have shown a strong correlation with acute myelomonocytic leukaemia with abnormal eosinophils [M4E] (77). Structural abnormalities of the long arm of chromosome 11 have been associated with acute monocytic leukaemia; in particular with the poorly differentiated form [M5a] found in children (6).

Some simple changes t[8;21] and inv[16] are reported to carry a relatively good prognosis, while others, affecting chromosomes 5 or 7, carry a poor prognosis [6]. A -5 and -7 chromosome abnormality is present in approximately 90% of patients with therapy-related ANLL, and is known to respond poorly to induction therapy (2,6,70).

The relationship between chromosomal abnormalities and clinical or blast cell features is three-fold, and includes those strongly associated with a particular FAB type, those associated with morphological features of the marrow which do not contribute to the FAB classification, and those which are found throughout ANLL (2).

**Table 6: Chromosomal abnormalities associated with FAB subtype or specific marrow morphology**

| Chromosome abnormalities | FAB type | Morphology |
|---|---|---|
| t[8;21] | M2 | Myeloblasts |
| t[15;17] | M3 | Promyelocytes |
| | M3 variant | Promyelocytes with microgranules |
| inv[16]<br>del[16] | M4E | Abnormal eosinophils |
| del or t[11][q23]<br>t[11][q13] | M5 | Monoblasts<br>Monocytoid |
| t[6;9] | Not specific | Increased basophils |
| inv[3] | | Thrombocytosis |
| t[3;3] | | Abnormal megakaryocytosis |

(2,4,5,6,70)

**Abnormalities found throughout ANLL**

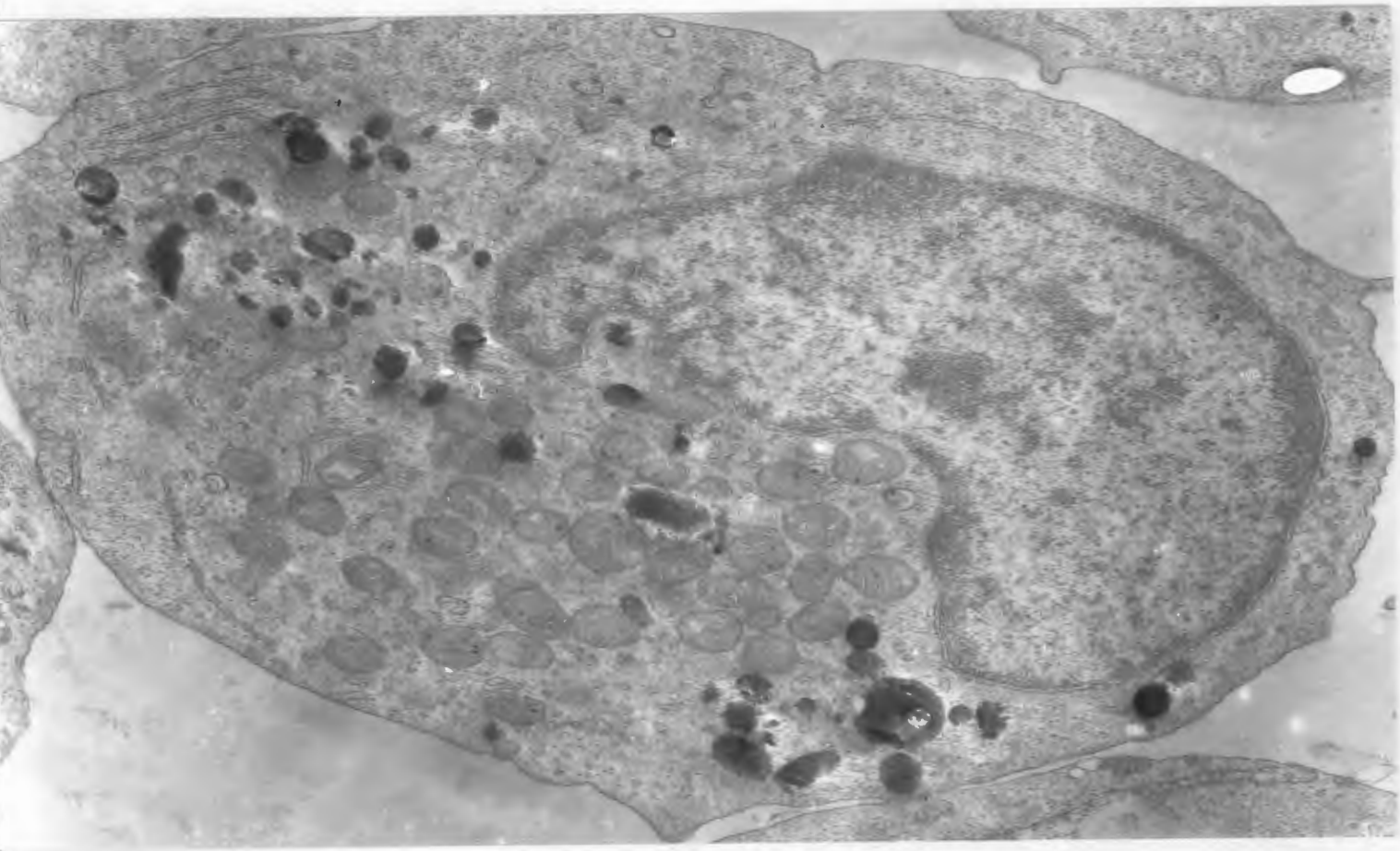
5 or 5q-
-7 or 7q-
5 and 7 abnormalities
+8
t/del 12p
+21

## ELECTRON MICROSCOPY

Electron microscopy [EM] can be invaluable in the classification of particular subtypes of ANLL, particularly M0 and M7. Peroxidase activity is present in the primary granules in blasts of the granulocytic series and shows reactivity in the nuclear membrane, endoplasmic reticulum, and Golgi region, and this can be used as a supportive investigation in cases of M0 (6). Peroxidase has also been shown in megakaryoblasts, except that neither granules nor the Golgi apparatus show peroxidase activity, and positivity is confined to the nuclear space and endoplasmic reticulum (6,79,88). Some

combined studies employing both platelets peroxidase and immunological staining for megakaryoblast surface glycoproteins has revealed that the platelet peroxidase positivity appears earlier than does the detection of CDw41 and CDw42 antigens (6).

**Figure 9:** **Electron microscopy with myeloperoxidase cytochemistry showing presence of myeloperoxidase in primary granules of myelocyte. [x25 000]**



**PROGNOSIS**

In the most recent MRC ANLL trial [1984-1988], the complete remission [CR] rate achieved with chemotherapy for those aged 60 or over was 41%, for those between 40 and 59 years 68%, and for those aged less than 40 years it was 83% (2).

Median survival time is now 12 months for ANLL patients, and for those who enter remission 25% are alive 24 months and 10% 60 months after remission induction (4).

In a current study at Groote Schuur Hospital [GSH], 58% of 106 ANLL patients achieved complete remission after their first induction therapy, with a median duration of remission being 38 months and with 39% of relapses occurring during the first 24 months.

Allogenic bone marrow transplantation [BMT] from a fully HLA-matched unreactive sibling offers an approximately 50% chance of long term disease-free survival [DFS] in children and young adults. In adults over the age of 40, long term DFS is not as good as it is in younger patients (2). Median post-bone marrow transplant survival at GSH is 65 months.

Features which have been associated with a poor prognosis in ANLL include: age over 60 years, a high peripheral white cell count, thrombocytopenia [less than 25 $x10^9$ /L] at presentation, chromosome abnormalities particularly involving chromosomes 5 and 7, (2,6) trilineage myelodysplasia, (60) and the presence of CD 34 positive blasts (82).

**TREATMENT**

A prerequisite for ANLL patients is induction of a profound bone marrow hypoplasia if they are to achieve complete remission (2). A combination of drugs is used which acts at different stages of the cell cycle, resulting in profound suppression of leukaemic cells and allowing recovery of normal haemopoietic cells (2,6).

Therapy in ANLL involves three stages - remission induction, consolidation and consideration of bone marrow transplant, and further intensification.

For remission induction three drugs are used; daunorubicin, cytosine arabinoside and thioguanine or etoposide. Daunorubicin and cytosine arabinoside are the two most important drugs for ANLL therapy. As the main problem facing ANLL patients in CR is the possibility of leukaemic relapse, remission induction is followed by consolidation

with further intensification by the use of, for example, mitozantrone in combination with cytosine arabinoside (2,100).

Recently, haematopoietic growth factors like granulocyte-macrophage colony stimulating factor [GM-CSF] have been given to patients before cytosine arabinoside, in order to stimulate proliferation of the leukaemic cells and to increase their sensitivity to chemotherapy, but there is as yet no evidence of an increase in remission rate or duration (100).

An alternative approach is to try to induce maturation of leukaemic cells. The most important clinical studies of differentiating agents involved persons with APL receiving all-trans retinoic acid with more than 90% achieving remission, and this was followed by post-remission chemotherapy (101,102,103).

In the Department of Haematology at GSH, treatment is divided into three phases, namely - remission induction with Fourth Cape Town Regimen [CTR IV], followed by consolidation with two courses of CTR IV and if possible by allogenic or autologous BMT.

Fourth Cape Town Regimen comprises the following agents: cytosine arabinoside, epipodophyllotoxin, VP16-213 and daunorubicin.
[The Cape Town Regimen for the Management of Acute Leukaemia. December. 1990]

# CHAPTER THREE

# MATERIALS AND METHODS

## PATIENT SELECTION

Thirteen cases of MDS and 16 cases of ANLL which had been diagnosed between May 1990 and July 1991 were evaluated. A decision was made to exclude patients with ALL, as cytochemistry does not play a major role in the diagnosis of this condition, and initial attempts with lymphoid monoclonal antibodies were unsuccessful.

At the time of bone marrow biopsy, patients had not received specific therapy and the initial diagnosis and classification was made on the basis of blood and bone marrow smears.

## LABORATORY PROCEDURES

**Blood smears from each patient were stained with Wright-Giemsa stain:**

**1. Wright-Giemsa stain**

| | | |
|---|---|---|
| Wright's stain powder | [Sigma, Lot 520728] | 9.0 g |
| Giemsa stain powder | [Merck, No 62410] | 1.0 g |
| glycerin | [Merck, No 9907229] | 90 ml |
| methanol [absolute] | [BDH, No R1680] | 2910 ml |

Reagents were mixed and stored in a brown bottle for 30 days prior to use.

**2. Phosphate buffer** [pH 6.4]

| | | |
|---|---|---|
| anhydrous monobasic potassium phosphate | [BDH, Lot 2266030L] | 6.63g |
| anhydrous dibasic sodium phosphate | [BDH, No 30343] | 2.56 g |
| distilled water | | 1000 ml |

**3. Methyl alcohol** [BDH, No 64186 H]

Air-dried blood smears were fixed with methanol for 30 seconds, flooded with Wright's stain for four minutes, and placed in a mixture of equal volumes of phosphate buffer and Wright's stain for seven minutes. They were rinsed with tap water and air dried (94).

Marrow aspirates - these were obtained by aspiration from the posterior superior iliac crest and stained with May-Grunwald-Giemsa.

May-Grunwald-Giemsa staining procedure:

**May-Grunwald stain** [Merck, Lot 832452]:
A 0,3% solution of May-Grunwald powder in methanol was prepared by grinding, filtered after 2-3 days, and diluted before use with a phosphate buffer solution of pH 7.2.

**Giemsa stain** [Merck, No 62410]:
0,6 gm Giemsa powder was added to 50 ml of methanol, and then 25 ml of glycerine was added. After 2-3 days, the solution was filtered and diluted before use with a phosphate buffer of pH 7.2 in 1:10 ratio.

Air-dried smears of blood or bone marrow were fixed for 15 minutes in methanol, transferred to diluted May-Grunwald solution for 15 minutes, and then transferred to diluted Giemsa solution for 30 minutes. The smears were washed in a phosphate buffer [pH 7.2], and air-dried (104).

In all patients, bone marrow biopsies were taken from the posterior superior iliac crest with a Jamshidi needle [gauge 8] and divided in half longitudinally in a specially designed holder. One half was used for paraffin processing, the other half for glycol methacrylate [GMA] embedding. Cores for GMA embedding were immediately placed

in 50% acetone at -20 °C for two hours for fixation and dehydration and then at -20 °C for two hours in Solution A [Sol.A] (24), which consisted of:

| | | |
|---|---|---|
| - hydroxyethyl-methacrylate | [Merck-Schuchardt, No 830K10514743] - | 100 ml |
| - 2-butoxyethanol | [Merck, No 9261276] | 15 ml |
| - benzoyl peroxide | [Merck-Schuchardt, No 8259168] | 1.0 g |
| - methyl benzoate. | [Merck, No 4563723] | 6 ml |

The reagents were mixed in a bottle which was placed on a rotary-mixer for 2-4 hours at 4 °C.

Embedding was done at -20 °C, in a mixture of 42 parts of Sol.A to one part of Solution B [Sol.B] (24), which consisted of:

| | |
|---|---|
| - polyethylene glycol 400 [BDH, No 4763820G] | 10 ml |
| - NN dimethylaniline [BDH, No 9497320K] | 1 ml |

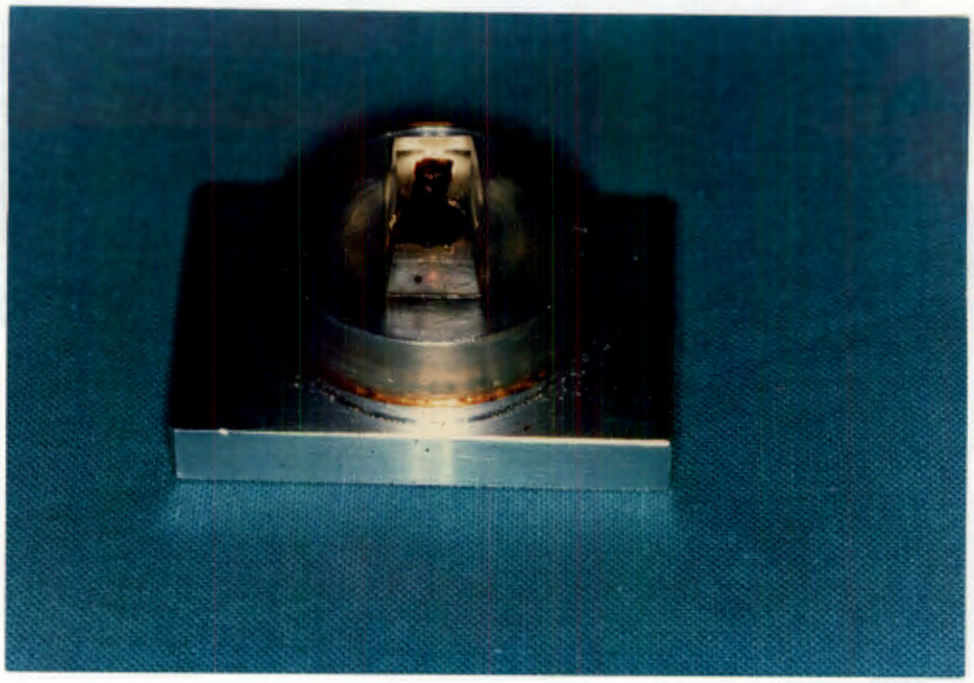
Marrow cores were then submerged in the embedding mixture of Sol.A and Sol.B in embedding moulds, placed at the top of the bath filled with a mixture of water and ethylene glycol cooled to a temperature of -20 °C. [Fig.10]

**Figure 10: Water bath with embedding moulds placed at the top**

2.5 ml of the embedding mixture was required to embed an 8-10 mm long marrow core.

The polymerization reaction is initially strongly exothermic and if it is allowed to proceed rapidly without adequate cooling, the boiling point of the monomer is exceeded. This results in the formation of gas bubbles which are trapped in the block. This may impair the morphological assessment and also the cytochemical and immunological reactions. By placing the specimens in a vacuum, which is essential for proper tissue infiltration, the boiling point of the embedding solution is further decreased. Polymerization was therefore performed at -20 $^{o}$C under a vacuum of 0.5 atmospheres for 4 hours and this proved to be effective for uniform and adequate polymerization [Fig.11] and for the preservation of cytochemical and immunological activity.

**Figure 11: A marrow core, embedded in GMA attached to aluminium block holder**



In a higher vacuum of more than 0,5 atmospheres, the polymerization reaction exceeded the boiling point of the embedding mixture with the formation of gas bubbles as previously described.

Sections were cut with disposable hardened steel knives on a 2040 Reichert-Jung microtome into 2 microns sections, floated in a water bath at 25 $^{o}$C, placed on slides and allowed to dry at room temperature. Sections not utilized immediately were stored in the freezer at -20 $^{o}$C for varying periods, ranging from a few weeks to a few months. They were processed in batches for cytochemistry or immunophenotyping.

All marrow sections were then routinely stained with:

**1. Haematoxylin and eosin:**

Haematoxylin [Harris haematoxylin] [Merck, Lot 09619162] was applied to sections for 4 minutes and washed off with water. Sections were differentiated for 2-3 seconds with 1% acid-alcohol, washed and left to blue in running tap water for 10 minutes.

Counterstain was then applied with 1% aqueous eosin [Gurr, No 21028] for 2 minutes at room temperature, and sections were then washed, air dried and mounted in DePeX mounting medium [Gurr, No 36125] (104).

**2. Enzymatic reactions:**

**Myeloperoxidase**

Sections were presoaked in normal saline for 15 minutes then preincubated for 10 minutes at room temperature in 5 mg DAB [3,3 diaminobenzidine tetra hydrochloride] [BDH, 4948480H ] and dissolved in 10 ml 0.05 M Tris buffer pH 7.6.

0.1 ml of 1% $H_2O_2$ [Lennon] was added to DAB-Tris solution and incubated for 20 minutes at room temperature.

Slides were rinsed three times with 0,05 M Tris buffer, counterstained with Harris haematoxylin for 5 minutes at 25 °C, air dried, and mounted in DePeX mounting medium (22).

**Chloroacetate esterase**

**Preparation of reagents:**

**1. Sol.A:**

0.01g alpha-naphthol AS chloroacetate [Sigma, Lot 105F - 5052] was dissolved in 0.5 ml N,N dimethylformamide. [Sigma, Lot 16C - 0050]

**2. Sol.B:**

0.1 ml New Fuchsin [Gurr, Lot 6840060N] was mixed with 0.1 ml 4% sodium nitrite [BDH, No 9909805] and left to react for 1 minute.
9.5 ml of a phosphate buffer of pH 7.6 was added.

Sol.A and Sol.B were mixed together and applied to sections for 1 hour at room temperature.
After incubation, sections were rinsed with water, counterstained with Harris haematoxylin for 5 minutes, air-dried, and mounted with DePeX mounting medium (22).

**Alpha-naphthyl butyrate esterase**

10 mg of alpha-naphthyl butyrate [Sigma, Lot 32H5002] was dissolved in 0.5 ml of ethylene glycol monomethyl ether [Merck, No 2048932] and 9.5 ml of 0.15 M phosphate buffer, pH 6.3 was added with 0,05 ml of pararosanilin [Sigma, Lot 118F-3709] working solution, which was made by mixing 1 gm of pararosanilin with 25 ml of 2 N HCl.

Sections were incubated in the above solution at 25 °C for 1.5 hour, rinsed with water, counterstained in Harris haematoxylin for 5 minutes at 25 °C, air dried and mounted with DePeX mounting medium (22).

**Alkaline phosphatase**

A stock solution was prepared by dissolving 30 mg naphthol AS phosphate [Sigma, Lot 77F - 5015] in 0.5 ml N,N-dimethylformamide [Sigma, Lot 16C - 0050] to which was added 100 ml of 0.2 M Tris buffer, pH 9.1.

10 mg of fast blue BB salt [Sigma, Lot 49F - 0040] was dissolved in 10 ml of stock solution. Sections were incubated for 1.5 hour at 25 °C, rinsed with water, counterstained with 0.1% aqueous neutral red [Gurr, No 23523] for 10 minutes at 25 °C, air dried and mounted with DePeX mounting medium (22).

**Acid phosphatase**

**Preparation of reagents:**

**1. Michaelis veronal-acetate buffer stock solution:**

| | | |
|---|---|---|
| sodium acetate-$3H_2O$ | [BDH, No 7438635] | 9.714 g |
| sodium barbiturate | [BDH, No 4811880H] | 14.714 g |
| distilled water | 500.00ml | |

**2. Incubation medium:**

| | |
|---|---|
| naphthol AS phosphate [ Sigma, Lot 100H - 5007] | 10 mg |
| dissolved in N,N-dimethylformamide | 1 ml |
| Michaelis stock solution | 5 ml |
| distilled water | 12 ml |
| 1 gm of pararosanilin dissolved in 25 ml 2 N HCL | 0.05 ml |

pH was adjusted to 6,3.

The incubation medium was applied at 37 °C for 60 minutes and, after incubation, sections were rinsed with distilled water. Sections were then counterstained with Harris haematoxylin for five minutes at 25 °C and mounted with DePeX mounting medium (22).

**Periodic acid Schiff**

**1. Periodic acid solution:**

| | |
|---|---|
| periodic acid crystals [BDH, No 5823930B] | 5 gm |
| distilled water | 500 ml |

**2. Schiff's basic fuchsin:**

| | |
|---|---|
| basic fuchsin [Gurr, Lot 6840060] | 5 gm |
| hot distilled water | 500 ml |

Filtered and saturated with $SO_2$ by bubbling for one hour.
Filtration was repeated through Whatman No.1 filter paper into a dark bottle.

Sections were incubated in 1% periodic acid for 10 minutes at 25 $^{o}$C, rinsed with distilled water and incubated in Schiff's reagent for 30 minutes at 25 $^{o}$C. After incubation, sections were washed in running tap water for 10 minutes, counterstained with Harris haematoxylin at 25 $^{o}$C, air-dried, and mounted with DePeX mounting medium (105).

In all enzymatic reactions positive and negative controls [peripheral blood smears] were stained simultaneously with GMA marrow sections.

**Gomori's Stain**

**Methenamine silver solution:**

| | |
|---|---|
| 3% methenamine [Sigma, Lot 68F0059] | 25.0 ml |
| distilled water | 25.0 ml |
| sodium tetraborate [BDH, No 0245370] | 2.0 ml |
| 5% $AgNO_3$ [BDH, No 221535] | 1.25 ml |

Sections were oxidized in 5% chromic acid [Merck, No CC580129] for one hour at room temperature, washed in tap water, then rinsed in sodium bisulphate [BDH, No

143104028] for one minute and again washed well in distilled water, and placed in methenamine silver solution at 56 °C for one hour.

At this stage slides were washed with warm water, toned in 0,1% gold chloride [SAARCHEM, No 19139] for 2-5 minutes, washed, fixed in sodium thiosulphate [SAARCHEM, No 18157] for 5 minutes and washed again in running tap water for 5 minutes.

Sections were counterstained in Light Green [Gurr, No 36829] solution for 30-40 seconds, washed in water and mounted in DePeX mounting medium (105).

**3. Prussian blue:**

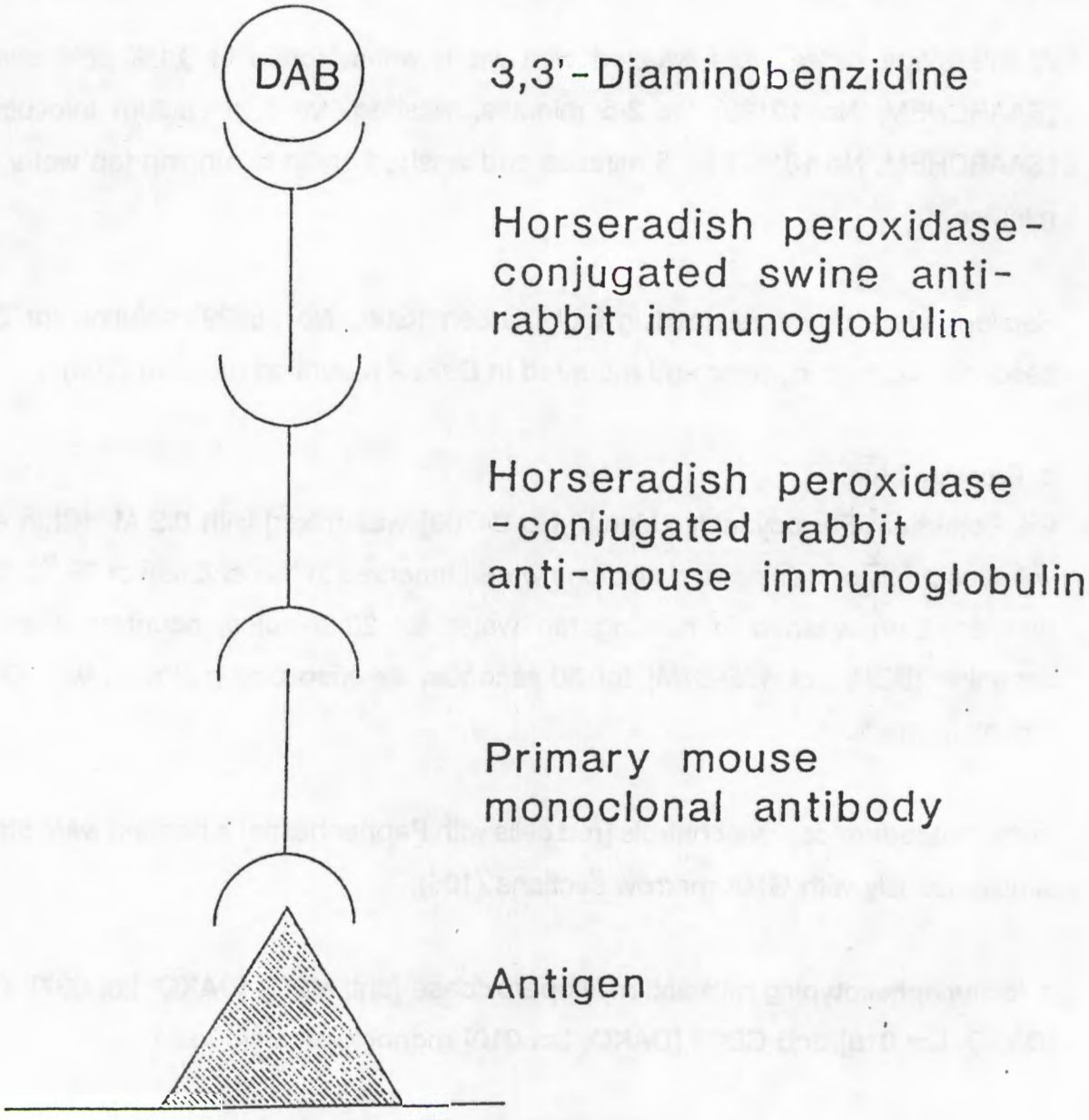
2% Potassium Ferrocyanide [Merck, No 24709] was mixed with 0.2 M HCl in equal volumes prior to staining and sections were immersed in this solution at 56 °C for 15 minutes, then washed in running tap water for 20 minutes, counterstained with safranine [BDH, Lot 426937M] for 30 seconds, air-dried and mounted with DePeX mounting medium.

In this procedure positive controls [red cells with Pappenheimer's bodies] were stained simultaneously with GMA marrow sections (105).

**4. Immunophenotyping** with anti-myeloperoxidase [anti-MPO] [DAKO, Lot 037], CD13 [DAKO, Lot 010] and CD33 [DAKO, Lot 010] monoclonal antibodies:

Immunohistologic staining of GMA sections was performed by the double-conjugate indirect peroxidase method modified for plastic sections (99).

See diagram below with accompanying methodology explanation.




Sections were allowed to hydrate for 15 minutes in 0.05M Tris-buffeted saline, [TBS] pH 7.6

Nonspecific binding of peroxidase was blocked by incubation with 2% normal swine serum in TBS for 30 minutes at 37 $^{o}$C.

Sections were then incubated with appropriate dilutions [1:100] of mouse monoclonal antibodies [DAKO] in TBS at 37 °C for 2 hours with anti-MPO antibody, and overnight at 4 °C with CD13 and CD33 monoclonal antibodies.

After two 5 minute washes in TBS, the sections were incubated in a 1/40 dilution of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin [DAKO, Lot 089] in TBS with 2.5% human AB serum for 1 hour at 37 °C.

After TBS washing, the sections were incubated in a 1/40 dilution of horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin [DAKO, Lot 030] in TBS with 2,5% human AB serum for 1 hour and subsequently washed in TBS at 37 °C. The peroxidase reaction was developed with 3,3'-diaminobenzidine 0.05% [DAB] [Sigma] in 0.05 M TBS, pH 7.6, with 0.1% $H_2O_2$ for 10 minutes at room temperature, washed in distilled water, counterstained with Harris haematoxylin for 5 minutes at 25 °C, blued in running tap water, air-dried, and mounted with DePeX mounting medium.

As a positive control GMA embedded marrow sections of a known case of ANLL were used, and these showed characteristic paranuclear positivity for myeloperoxidase.

Negative controls [no primary antibody applied] were processed simultaneously with GMA marrow sections.

**MORPHOLOGIC CRITERIA**

In each case (for both cytology and biopsies), 500 cells were counted to establish a differential count of blasts, myeloid and erythroid precursors, lymphocytes, plasma cells and the myeloid/erythroid ratio.

In addition, the percentage of ring sideroblasts was established by counting 100 nucleated cells on Prussian blue stained samples.

In each case the following features were evaluated:
number of blasts, cellularity, signs of dyserythropoiesis [multinuclearity, nuclear fragments, cytoplasmic abnormalities, number of erythroblasts [<5%, >60%] and of ring sideroblasts [>15%], dysmegakaryopoiesis, [micromegakaryocytes, large mononuclear forms, multiple small nuclei], dysgranulopoiesis, [nuclear abnormalities - pseudo Pelgar-Huet forms, hypogranular cells] and the number of monocytes in the peripheral blood.

Architectural distortion of the marrow was also assessed in MDS and cytochemical staining was applied in all patients and immunophenotypic in selected cases.

The following criteria were used for the grading of collagen reticulin in marrow sections: (106)

| | |
|---|---|
| Grade N or 1+ | No or occasional fine collagen fibres only. |
| Grade 2+ | Fine collagen fibres forming a network throughout most of the sectors. |
| Grade 3+ | Diffuse fibre network with scattered thick coarse fibres. |
| Grade 4+ | Diffuse, often coarse fibres with collagenization. |

The cellularity of aspirate and biopsy samples was assessed as below:

| | |
|---|---|
| 10-25% | grossly hypocellular. |
| 25-40% | mildly hypocellular. |
| 40-60% | normocellular. |
| 60-90% | mildly hypercellular. |
| >90% | grossly hypercellular. |

Criteria used for grading iron stains in bone marrow aspirates: (107)

| | |
|---|---|
| Grade 0 | No iron granules observed. |
| Grade 1+ | Small granules in reticulum cells, seen only with oil-immersion lens. [x400] |
| Grade 2+ | Few small granules visible with low-power lens. [x100] |
| Grade 3+ | Numerous small granules in all marrow particles. |
| Grade 4+ | Large granules in small clumps. |
| Grade 5+ | Dense, large clumps of granules. |
| Grade 6+ | Very large deposits, obscuring marrow detail. |

Criteria used for grading iron stores in bone marrow GMA embedded sections:

| | |
|---|---|
| Grade 0 | No iron granules observed. |
| Grade 1+ | Single, occasional iron granules seen in reticulum cells. |
| Grade 2+ | Iron granules easily visible in reticulum cells with low-power lens. [x100] |
| Grade 3+ | Numerous small iron granules seen in reticulum cells. |
| Grade 4+ | Large iron granules seen in reticulum cells. |
| Grade 5+ | Reticulum cells are heavily laden with iron granules. |
| Grade 6+ | Reticulum cells are numerous and heavily laden with large iron granules, obscuring marrow detail. |

# CHAPTER FOUR

# RESULTS

## PATIENT DATA

Thirteen cases of MDS and 16 of ANLL diagnosed between May 1990 and July 1991 were evaluated prior to treatment.

MDS patients consisted of RA [2], RARS [2], RAEB [2], RAEB-t [3] and CMML [4].

ANLL included M0[1], M1[6], M2[3], M3[2] M4[1], M4E[1], M6[1]. One trephine biopsy from this group could not be assessed as it was extensively necrotic.

# LABORATORY DATA

I

## MDS PATIENTS

Of the 13 cases one was female and twelve were male, with a median age of 68 years, the age range being 35-74 years.

12 patients were anaemic [haemoglobin less than 120 g/L] with a range of 56-130, 7 were leukopenic [white cell count less than 4 $x10^9$ /L] with a range of 1,2-38 and 6 were thrombocytopenic [platelet count less than 140 $x10^9$ /L] with the range being between 13 and 680.

An increased monocytic component in the peripheral blood [more than 1 $x10^9$ /L] was noted in 4 subjects [No's 3, 4, 9, 12].

Two patients had markedly increased blast counts in the peripheral blood, and Auer rods were noticed in only one case [see Table 7].

**Table 7: Age, sex, and presenting haematological peripheral blood indices**

| Patient No | Sex | Age years | Hb g/L | WCC $x10^9$ /L | Mon % | Plt $x10^9$ /L | Blasts % | Auer rods |
|---|---|---|---|---|---|---|---|---|
| 1. | M | 72 | 103 | 4,0 | 15 | 13 | 3 | - |
| 2. | M | 67 | 83 | 4,7 | 1 | 191 | - | - |
| 3. | M | 70 | 101 | 8,8 | 30 | 195 | - | - |
| 4. | M | 67 | 107 | 3,8 | 28 | 165 | - | - |
| 5. | M | 68 | 130 | 1,5 | 6 | 226 | - | - |
| 6. | M | 54 | 87 | 2,6 | 7 | 17 | 1 | - |
| 7. | M | 53 | 89 | 1,5 | 2 | 39 | 3 | - |
| 8. | M | 65 | 97 | 3,0 | 9 | 27 | - | - |
| 9. | F | 74 | 56 | 4,8 | 22 | 285 | - | - |
| 10. | M | 68 | 82 | 1,2 | 4 | 75 | 46 | - |
| 11. | M | 35 | 60 | 2,4 | 6 | 680 | - | - |
| 12. | M | 73 | 74 | 38,0 | 13 | 178 | - | - |
| 13. | M | 39 | 102 | 8,9 | 2 | 43 | 35 | + |

**Abbreviations:** Hb - haemoglobin, WCC - white cell count, Mon - monocyte count, Plt - platelet count, + present, - not present.

**ARCHITECTURAL DISTORTION**

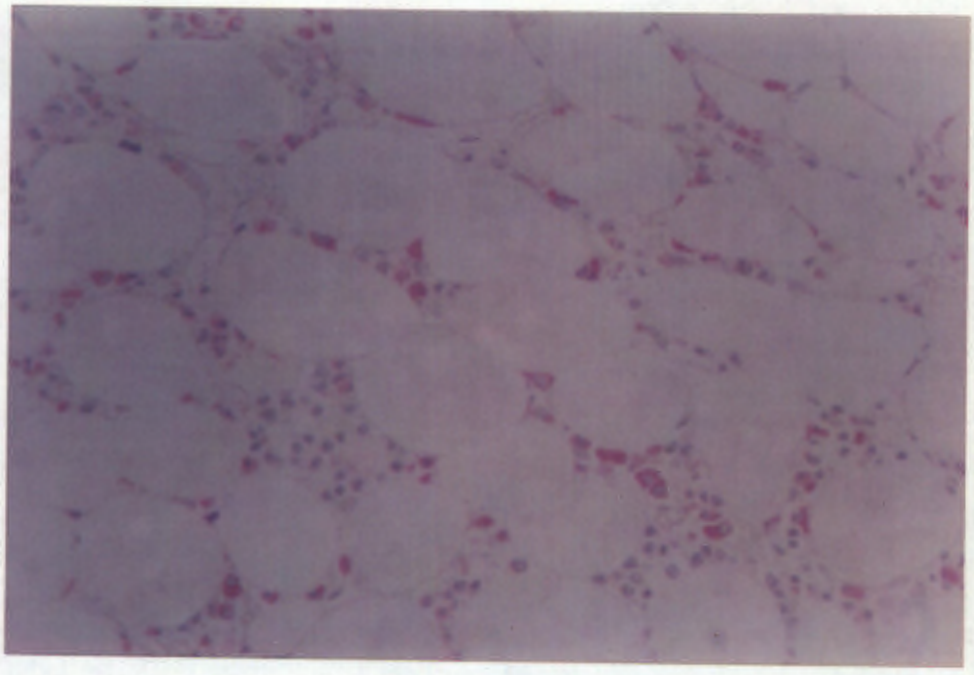
Normally, marrow erythroid precursors are present intertrabecularly in small clusters, in different stages of maturation. Early myeloid precursors are in proximity to the endosteal surfaces of trabeculae and to arterial vessels, whereas mature forms are scattered throughout the central intertrabecular areas. A distortion of this topographic distribution is found in several diseases, but typically in MDS. Distortion of normal marrow architecture was observed in 8 cases : [No's 1, 2, 6, 7, 8, 11, 12, 13]; whereas

abnormal localization of immature precursors [ALIP] was noticed on biopsy in four cases. [No's 6, 10, 11, 13] [Fig.6]

**CELLULARITY**

In 11 cases marrow sections were hypercellular, in one case it was normocellular, and hypocellular in one [Fig.12].

**Figure 12: Hypocellular marrow in MDS, showing chloroacetate esterase activity in myeloid precursors. [x100] GMA**



Cellularity was found to be similar on marrow sections and on marrow smears in 8 out of 13 cases. Aspiration failed in two cases. [No's 6, 10] [see Table 8].

**Table 8: Bone marrow cellularity on aspirate [BMA] versus biopsy sections [BMB].**

| | 10<25% | 25<45% | 45<60% | 60<90% | 90<100% | Total[BMA] |
|---|---|---|---|---|---|---|
| **DT** | 1 | | | 1 | | 2 |
| **10<25%** | **0** | | | | | 0 |
| **25<45%** | | **0** | | | | 0 |
| **45<60%** | | | **1** | | | 1 |
| **60<90%** | | | | **6** | 3 | 9 |
| **90<100%** | | | | | **1** | 1 |
| **Total[BMB]** | 1 | 0 | 1 | 7 | 4 | 13 |

**Abbreviations:**

BMB - bone marrow biopsy

BMA - bone marrow aspirate

DT - dry tap

**BLASTS**

Agreement in blast counts was observed in only 7 out of 13 cases, with marrow sections showing higher counts, resulting in a change of diagnosis and prognosis in six patients.

Two aspirates failed [No's 6, 10], and the blast count in one of these patients on paraffin sections was extended to be 10%, as against 60% on GMA sections.

Biopsy blast counts in two subjects was higher than 30%, resulting in a diagnosis of ANLL. [No's 10, 13] In two cases of CMML [No's 4, 12] the diagnosis was changed on biopsy sections to RAEB, and in two further patients [No's 6, 11] an alteration was made from RA and RARS respectively to RAEB [see Table 9].

**Table 9: Blast infiltration on aspirate [BMA] versus marrow biopsies [BMB]**

| | <5% | 5-20% | 20-30% | >30% | Total[BMA] |
|---|---|---|---|---|---|
| **DT** | | 1 | | 1 | 2 |
| **<5%** | 4 | 3 | | | 7 |
| **5-20%** | | 2 | | 1 | 3 |
| **20-30%** | | | 1 | | 1 |
| **>30%** | | | | 0 | 0 |
| **Total [BMB]** | 4 | 6 | 1 | 2 | 13 |

**Abbreviations:**

BMB - marrow sections

BMA - marrow aspirate

DT - dry tap

**DYSHAEMOPOIESIS**

Marrow aspirate failed in two patients. Quantitative and qualitative changes in erythropoiesis were as easily assessed on aspirates as on marrow sections, although in four cases aspirates did not show dyserythropoiesis. Quantitative and qualitative features of dysmyelopoiesis and dysmegakaryopoiesis were more apparent on marrow biopsies [see Table 10].

**Table 10:** **Summary of the differences in dyshaemopoiesis between smears and marrow sections**

| Red cell series | Biopsy | Aspirate |
|---|---|---|
| Quantitatively disturbed | 7 | 7 |
| Dyserythropoiesis | 9 | 8 |
| Normal | 0 | 4 |
| **White cell series** | | |
| Quantitatively disturbed | 7 | 4 |
| Dysmyelopoiesis | 10 | 6 |
| Normal | 0 | 5 |
| **Megakaryocytic series** | | |
| Quantitatively disturbed | 9 | 3 |
| Dysmegakariopoiesis | 13 | 7 |
| Normal | 0 | 5 |

**Table 11:** **Summary of morphological features found in aspirates and the GMA-embedded sections**

| Patient Cellularity | | | Blasts | | RS | | DysE | | DysG | | DysM | | Diagnosis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | A | H | A | H | A | H | A | H | A | H | A | H | A [AH] H |
| 1. | 80% | 80% | 25% | 23% | ND | - | + | + | + | + | + | + | RAEB-t |
| 2. | 70% | 90% | <5% | <5% | 15% | PS | + | + | - | - | + | + | RA |
| 3. | 60% | 80% | 4% | 4% | - | ND | - | - | - | - | + | + | CMML |
| 4. | 70% | 95% | <5% | 16% | ND | - | + | + | + | + | + | + | CMML CMML/RAEB |
| 5. | 60% | 80% | 2% | 0% | >15% | >15% | + | + | + | + | - | + | RARS |
| 6. | DT | 60% | DT | 8% | DT | >15% | DT | + | DT | + | DT | + | RA RAEB |
| 7. | 50% | 50% | 10% | 11% | PS | <15% | + | + | - | - | - | + | RAEB |
| 8. | 70% | 60% | 7% | 6% | - | <15% | - | + | + | + | - | + | RAEB |
| 9. | 70% | 70% | 4% | 4% | >15% | >15% | + | - | + | + | + | + | CMML/RARS |
| 10. | DT | 10% | DT | 60% | DT | - | DT | - | DT | + | DT | + | [RAEB-t] ANLL |
| 11. | 70% | 90% | 2% | 20% | >15% | <15% | + | + | - | + | + | + | RARS RAEB |
| 12. | 80% | 75% | 0% | 16% | - | - | - | - | - | + | - | + | CMML CMML/RAEB |
| 13. | 90% | 90% | 18% | 32% | PS | - | + | + | + | + | + | + | RAEB-t ANLL |

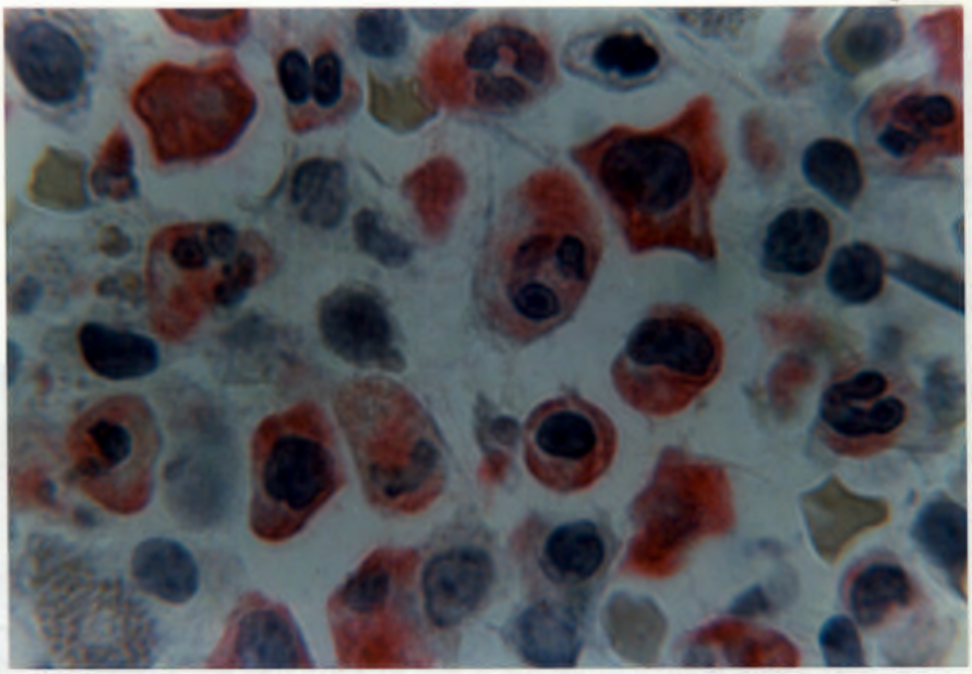
**Abbreviations:**

A - aspirate, H - histology, [AH] - aspirate and histology, + present, - not present, PS - pathological sideroblasts, RS - ring sideroblasts, DysE - dyserythropoiesis, DysG - dysgranulopoiesis, DysM - dysmegakaryopoiesis, DT - dry tap, [RAEB-t] - diagnosis made on marrow paraffin sections.

## CYTOCHEMICAL FINDINGS

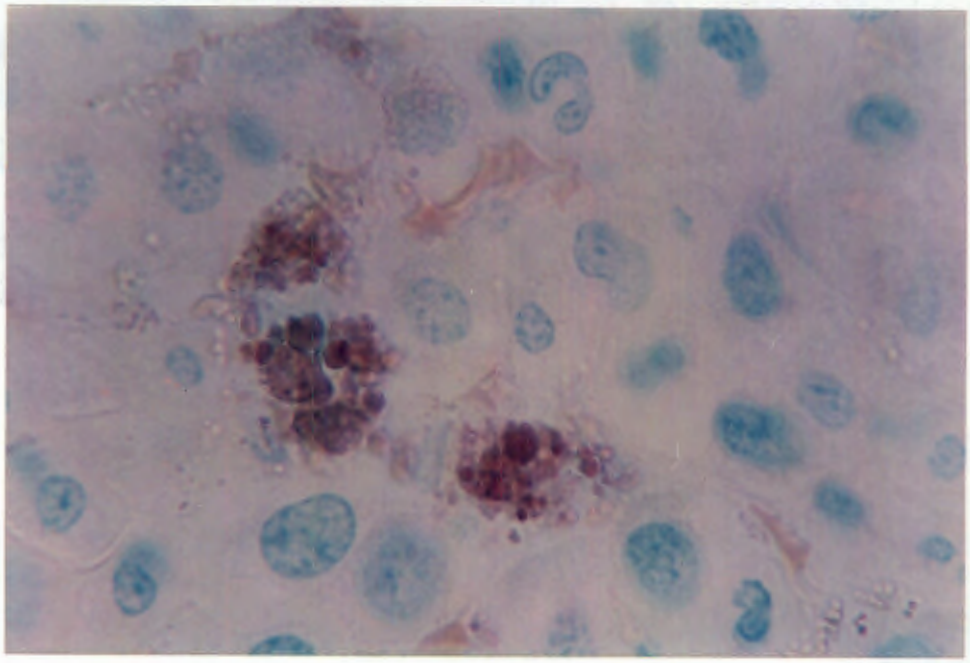
Chloroacetate esterase was found to be a very sensitive marker of myeloid cells and also mast cells, which appeared to be very prominent in several cases. This stain can also be used to demonstrate dysplasia in myeloid precursors as it shows uneven cytoplasmic staining [Fig.13].

**Figure 13:** **Chloroacetate esterase from MDS case showing myeloid cells at different stages of maturation, with uneven cytoplasmic staining in some of the cells. [x400] GMA**



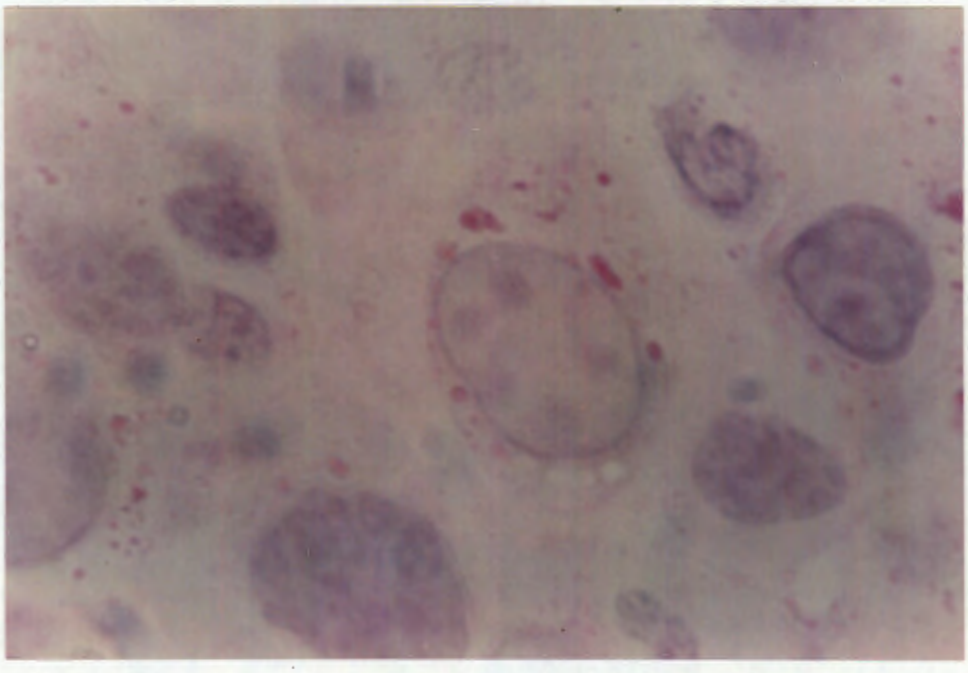
Alpha-naphthyl butyrate esterase did not stain the monocytic component in GMA-embedded sections, showing positivity only in the reticulum cells [Fig.14].

**Figure 14:** **Alpha-naphthyl butyrate esterase showing positivity in an elongated, large reticulum cell. [x400] GMA**



PAS stain showed distinct block positivity in erythroid precursors in the RAEB-t case, which was transforming into acute leukaemia- M6 [case No.1] [Fig.15].

**Figure 15:** **PAS cytochemistry from a case of RAEB - transforming into erythroleukaemia showing block positivity in the cytoplasm of abnormal erythroblasts. [x1000] GMA**



Acid phosphatase positivity was very distinct in osteoclasts [Fig.16] and reticulum cells [Fig.17]. It can therefore be a good marker of their activity in the marrow.

**Figure 16:** **Acid phosphatase cytochemistry from a case of MDS showing distinct cytoplasmic positivity in osteoclasts. [x1000] GMA**

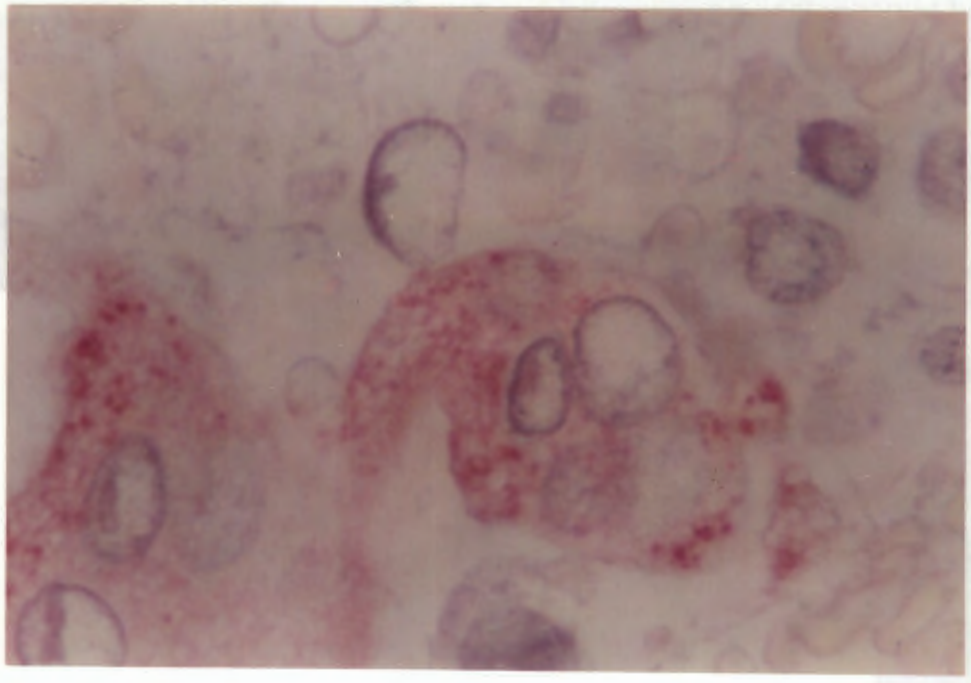
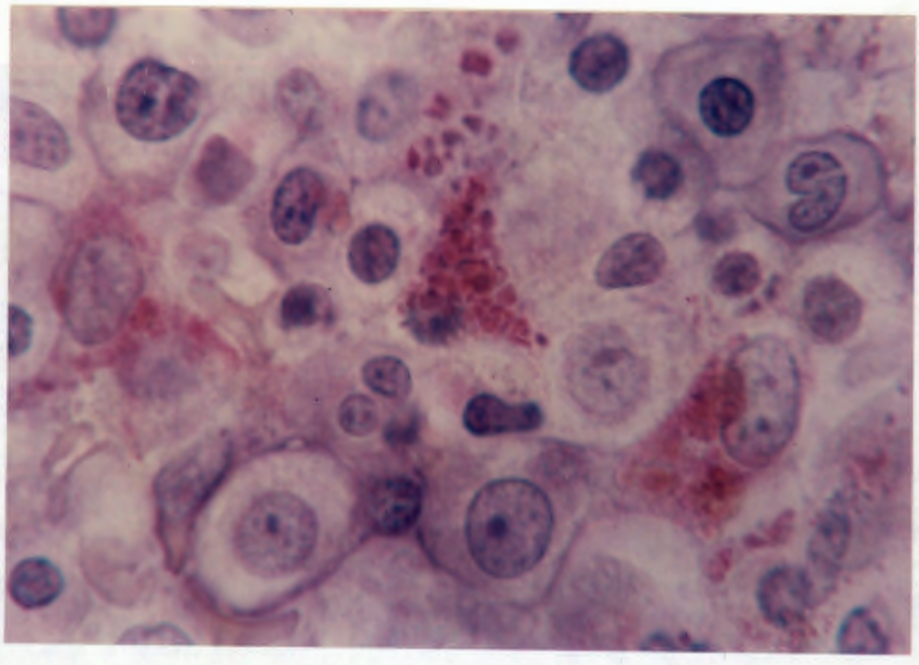


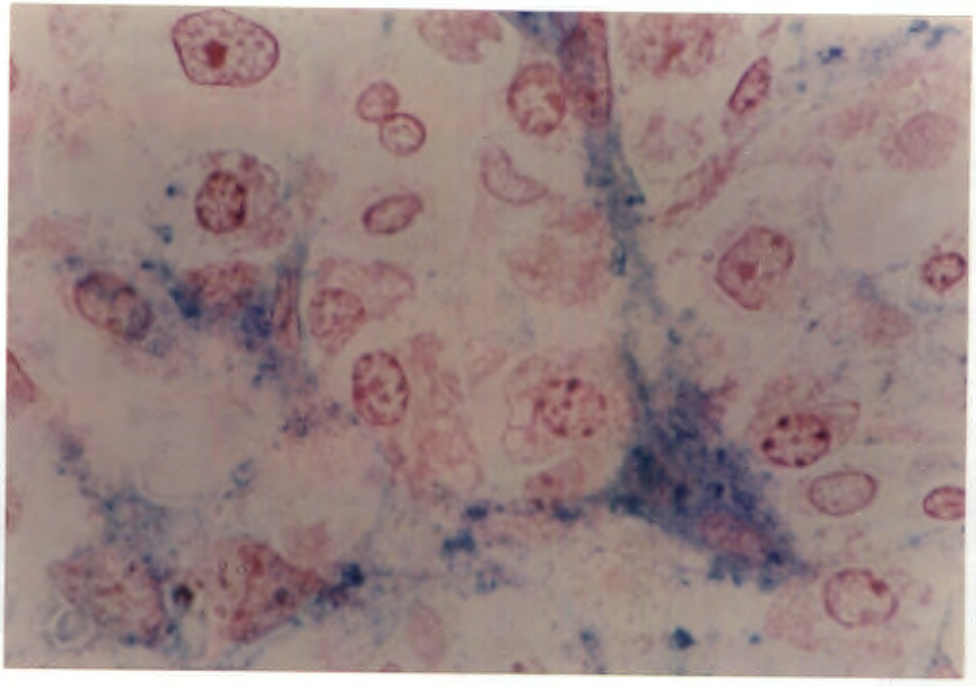
**Figure 17:** **Acid phosphatase cytochemistry from MDS case showing strong positivity in reticulum cells. [x600] GMA**



Gomori's stain was unsuccessful as a stain for reticulum in GMA-embedded marrow sections [no reticulum was shown even in cases with marked increases in reticulum in routine paraffin sections].

Alkaline phosphatase appeared to be a very sensitive stain for reticulum as well as for alkaline phosphatase positive reticulum cells [AL-RC], (22) which in some cases were very prominent [Fig.18].

**Figure 18:** **Alkaline phosphatase cytochemistry from a case of MDS showing presence of alkaline phosphatase positive reticulum cells [AL-RC] [x1000] GMA**



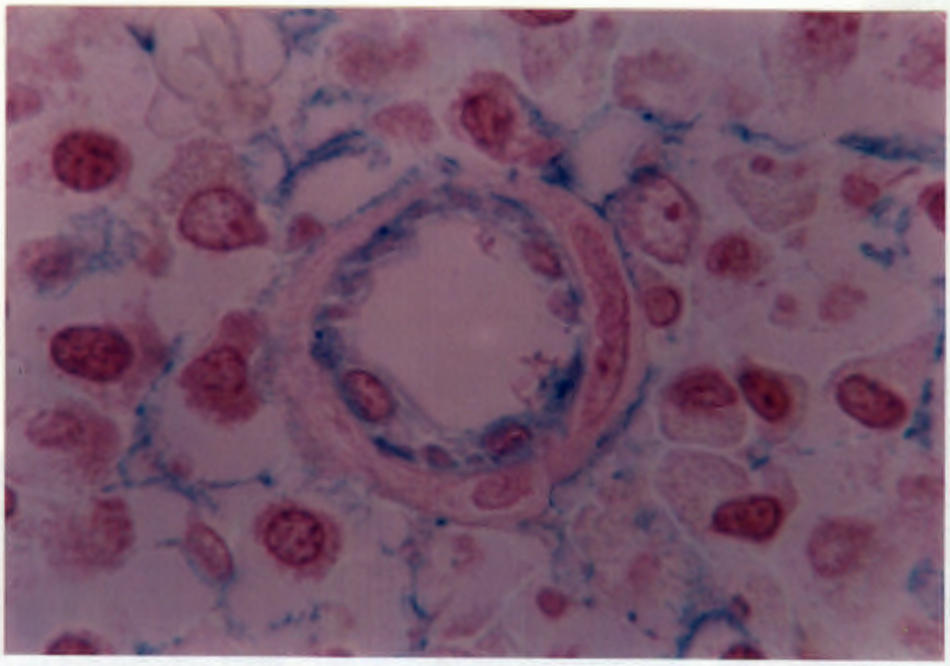
Recently it has became evident that two cell types seem to be of particular importance: one is a non-phagocytic cell [AL-RC], which stains for alkaline phosphatase, is dendritic in shape [Fig.18], and closely associated with granulocytic precursors (108). The other is a macrophage commonly associated with erythroid precursors.

Dilly and Jagger (108) reported markedly increased numbers of AL-RC cells in all studied cases of ANLL and severe reduction of AL-RC in ALL. An increase in their numbers was observed in some cases of MDS when they were also associated with

an increase in reticulum (22). An increase in the number of AL-RC was observed in 8 individuals [in some this was very prominent].

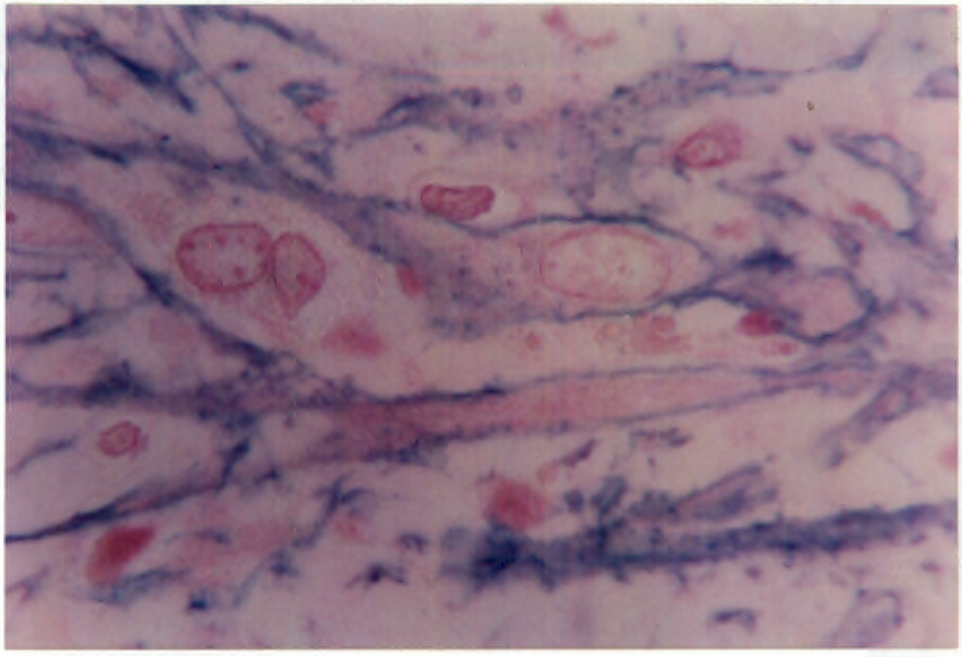
Several studies reported that chondrocytes, osteoblasts, and endothelial cells of small arterioles [Fig.19] are also reactive with alkaline phosphatase (22,108).

**Figure 19:** **Alkaline phosphatase cytochemistry from a case of ANLL showing positivity in endothelial cells of a small arteriole. [x600] GMA**



Alkaline phosphatase was performed in all except two cases, and in cases No's 1, 2, 5, 7, 10, 11, 12, 13 showed marked increases in reticulum [1+ or more] [Fig.20].

**Figure 20: Alkaline phosphatase cytochemistry from an MDS case showing markedly increased reticulum [x400] GMA**

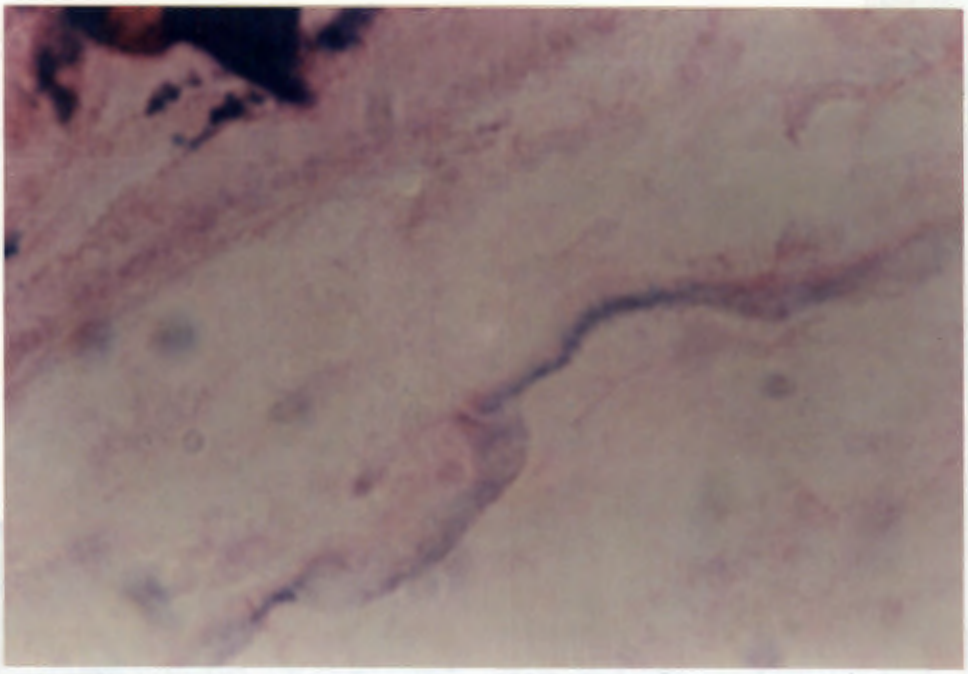


**IRON STUDIES**

GMA sections showed good quality iron staining with Prussian blue [Fig.21], and this, together with excellent morphology, allowed for easy and accurate detection of ring and pathological sideroblasts [Fig.22].

It also made it possible to detect the presence of stainable iron at the surface of trabecula bone in RARS cases [Fig.23].

**Figure 23: Prussian blue stain from a case of RARS showing the presence of stainable iron at the surface of trabecula bone. [x1000] GMA**



The amount of iron present in marrow aspirate particles is comparable to the iron that is in reticulum cells on marrow sections, with only slight differences, but full concordance was observed in only four subjects [see Table 12].

More than 15% of RS was observed on both the aspirate and biopsy in cases 5 and 9, with the former showing the higher percentage. In case 11 only occasional RS were detected on marrow sections, while the aspirate showed 15% of these cells. In case 8 occasional RS were detected only on the marrow sections. The sections showed the presence of 49% RS, while the aspirate failed in patient 6. Aspirate also failed in case No 10 [see Table 12].

**Figure 21:** **Prussian blue stain from a case of RARS showing reticulum cells heavily laden with iron. [x400] GMA**

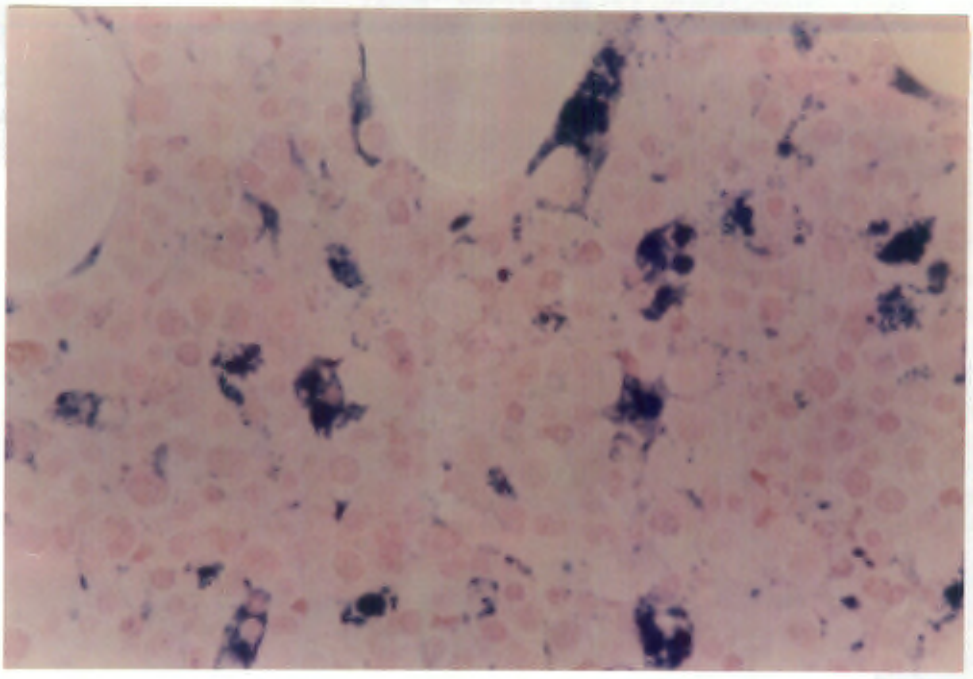


**Figure 22:** **Prussian blue stain from a case of RARS showing the presence of ringed sideroblasts. [x1000] GMA**

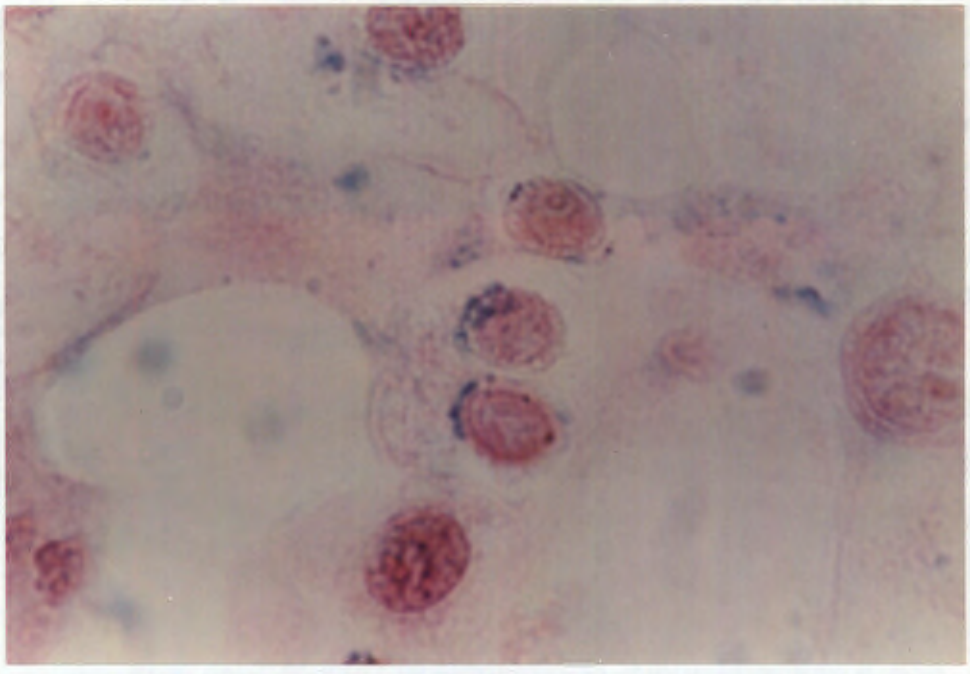


**Table 12:**

| Patient No | A | H |
|---|---|---|
| 1 | ND | 2/6 |
| 2 | 1/6 | 1/6 PS |
| 3 | 1/6 | ND |
| 4 | ND | 1/6 |
| 5 | 4/6 58%RS | 5/6 41%RS |
| 6 | DT | 5/6 49%RS |
| 7 | 4/6 PS | 2/6 <15%RS |
| 8 | 0/6 | 0/6 <15%RS |
| 9 | 5/6 50%RS | 3/6 26%RS |
| 10 | DT | 3/6 |
| 11 | 4/6 15%RS | 3/6 OCRS |
| 12 | 1/6 | 1/6 |
| 13 | 4/6 | 4/6 |

**Abbreviations:**

A - aspirate, H - histology, ND - not done, RS - ring sideroblasts, OCRS -occasional ring sideroblasts, PS - pathological sideroblasts, DT - dry tap.

**CYTOGENETIC ABNORMALITIES**

Six out of thirteen patients had karyotypic abnormalities. Two showed 5q- abnormality, which is characteristic for MDS and is common in all subtypes except CMML. The t[8;21] translocation observed in case 13 is unusual for MDS and is predominantly present in M2-ANLL (2,84) [see Table 13].

**Table 13:**

| Patient No | Cytogenetic abnormality |
|---|---|
| 1 | 5q-, 7q-, 9q-, 17p+, |
| 6 | 1p-, 2p+, t[5q;12q], t[2q;3p] |
| 8 | -16, t[1;16] |
| 10 | 46, XY, 5q- |
| 11 | 47, XY, +21 |
| 13 | t[8;21] |

**FINAL DIAGNOSTIC CONCORDANCE**

Agreement in a final morphological diagnosis was observed in 7 out of 13 patients. In two out of the six discordant cases histopathological diagnosis was already ANLL-M2, and in the remaining four cases, the morphological diagnosis was RAEB as opposed to RA, RARS, and two cases of CMML [see Table 14].

**Table 14: Concordance between marrow aspirate [BMA] and marrow biopsy [BMB]**

| | RA | RARS | RAEB | RAEB-t | CMML | ANLL | Total[BMA] |
|---|---|---|---|---|---|---|---|
| **RA** | 1 | | 1 | | | | 2 |
| **RARS** | | 1 | 1 | | | | 2 |
| **RAEB** | | | 2 | | | | 2 |
| **RAEB-t** | | | | 1 | | 2 | 3 |
| **CMML** | | | 2 | | 2 | | 4 |
| **ANLL** | | | | | | 0 | 0 |
| **Total [BMB]** | 1 | 1 | 6 | 1 | 2 | 2 | 13 |

**Abbreviations:**

BMB - marrow sections

BMA - marrow aspirate

II

**ANLL PATIENTS**

There were 8 females and 8 males with a median age of 44 years, the range being between 17 and 85 years. All patients were anaemic at presentation [Hb less than 120 g/L] with a range of 45-114. Ten had a WCC higher than 10 $x10^9$ /L, 5 had WCC less than 4 $x10^9$ /L and all 16 patients were thrombocytopenic with platelet counts of less than 140 $x10^9$ /L, the range being between 14 and 135.

An increased monocytic component in the peripheral blood [more than 5 $x10^9$ /L] was noted in two patients.

The percentage of blasts in the peripheral blood varied from 3% to 96%, and Auer rods were found in 9 subjects [see Table 15].

**Table 15: Age, sex, and presenting haematological peripheral blood indices**

| Patient No | Sex | Age years | Hb g/L | WCC $x10^9$ /L | Mon % | Plt $x10^9$ /L | Blasts % | Auer rods |
|---|---|---|---|---|---|---|---|---|
| 1. | M | 17 | 75 | 0,9 | 0 | 93 | 6 | - |
| 2. | F | 46 | 87 | 145,0 | 0 | 96 | 96 | + |
| 3. | F | 30 | 63 | 1,7 | 1 | 135 | 66 | - |
| 4. | F | 57 | 68 | 32,5 | 54 | 41 | 5 | + |
| 5. | F | 46 | 81 | 178,8 | 3 | 14 | 86 | + |
| 6. | F | 85 | 76 | 31,5 | 0 | 36 | 45 | - |
| 7. | M | 17 | 59 | 18,5 | 2 | 81 | 83 | - |
| 8. | M | 56 | 102 | 2,5 | 14 | 80 | 48 | + |
| 9. | M | 23 | 112 | 22,4 | 1 | 35 | 68 | + |
| 10. | M | 44 | 92 | 77,9 | 0 | 35 | 90 | + |
| 11. | F | 58 | 72 | 3,9 | 0 | 46 | 80 | + |
| 12. | M | 50 | 101 | 5,3 | 5 | 48 | 68 | + |
| 13. | F | 34 | 114 | 10,3 | 1 | 26 | 31 | - |
| 14. | M | 49 | 77 | 308,8 | 11 | 33 | 89 | - |
| 15. | F | 25 | 45 | 40,0 | 10 | 39 | 87 | + |
| 16. | M | 40 | 62 | 2,6 | 10 | 41 | 3 | - |

**Abbreviations:** Hb - haemoglobin, WCC - white cell count, Mon - monocyte count, Plt - platelet count, + present, - not present.

**CELLULARITY**

In almost all cases, bone marrow cellularity in biopsy sections was concordant with that on the aspirate, with the exception of two cases in which the aspirate showed slightly higher cellularity [see Table 16].

**Table 16: Bone marrow cellularity on aspirate [BMA] and biopsy sections [BMB]**

| | 10<25% | 25<45% | 45<60% | 60<90% | 90<100% | Total[BMA] |
|---|---|---|---|---|---|---|
| 10<25% | 0 | | | | | 0 |
| 25<45% | | 0 | | | | 0 |
| 45<60% | | | 0 | | | 0 |
| 60<90% | | 1 | | 3 | | 4 |
| 90<100% | | | | 1 | 10 | 11 |
| Total[BMB] | 0 | 1 | 0 | 4 | 10 | 15 |

**Abbreviations:**

BMB - bone marrow biopsy

BMA - bone marrow aspirate

**BLASTS**

In 7 cases the marrow aspirate blast count was higher than that estimated on the marrow sections, while eight cases showed similar counts on both aspirate and marrow sections [see Table 17].

**Table 17: Blast infiltrate on aspirate [BMA] and biopsy sections [BMB]**

| | <20% | 20<40% | 40<60% | 60<80% | >80% | Total[BMA] |
|---|---|---|---|---|---|---|
| <20% | 0 | | | | | 0 |
| 20<40% | | 0 | | | | 0 |
| 40<60% | | | 3 | | | 3 |
| 60<80% | | 1 | 1 | 0 | | 2 |
| >80% | | 1 | 2 | 2 | 5 | 10 |
| Total[BMB] | 0 | 2 | 6 | 2 | 5 | 15 |

**Abbreviations:** BMB - bone marrow biopsy
BMA - bone marrow aspirate

**DYSHAEMOPOIESIS**

Dysplastic features were not prominent in any patients, except cases No's 4, 12, and 16 [see Table 18].

**Table 18: Summary of morphological features present in marrow aspirate and biopsies**

| Patient | Cellular | | Blasts | | RS | | DysE | | DysG | | DysM | | Diagnosis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | A | H | A | H | A | H | A | H | A | H | A | H | A [AH] H |
| 1. | 90% | 95% | 90%P | 95%P | - | - | + | + | - | - | - | - | M3 |
| 2. | 90% | 90% | >90% | 80% | - | - | - | - | - | - | - | - | M1 M2 |
| 3. | 90% | 95% | >90% | 90% | - | - | - | - | - | - | - | + | M0 |
| 4. | 80% | 90% | 80% | 32% | - | - | + | - | - | - | + | + | M4 |
| 5. | 90% | 90% | 85% | 90% | - | - | - | - | - | - | - | - | M1 |
| 6. | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | |
| 7. | 90% | 100% | 86% | 76% | - | - | - | + | - | - | - | - | M1 |
| 8. | 90% | 95% | 95%P | 82%P | - | - | - | - | - | - | - | - | M3 |
| 9. | 90% | 90% | 70% | 47% | - | PS | - | + | + | - | - | - | M2 |
| 10. | 90% | 70% | 90% | 55% | - | <15% | - | - | - | - | - | - | M1 M2 |
| 11. | 70% | 70% | 86% | 70% | - | - | - | - | - | - | - | - | M1 M2 |
| 12. | 60% | 40% | 43% | 53% | - | <15% | - | + | + | + | - | - | M2 |
| 13. | 80% | 70% | 51% | 55% | - | - | - | - | + | + | - | - | M2 |
| 14. | 90% | 100% | 88% | 33% | - | - | - | - | - | + | - | - | M4E |
| 15. | 90% | 100% | 87% | 50% | - | - | - | - | - | + | - | - | M1 M2 |
| 16. | 70% | 80% | 50% | 45% | - | - | + | + | + | - | - | + | M6 |

**Abbreviations:**

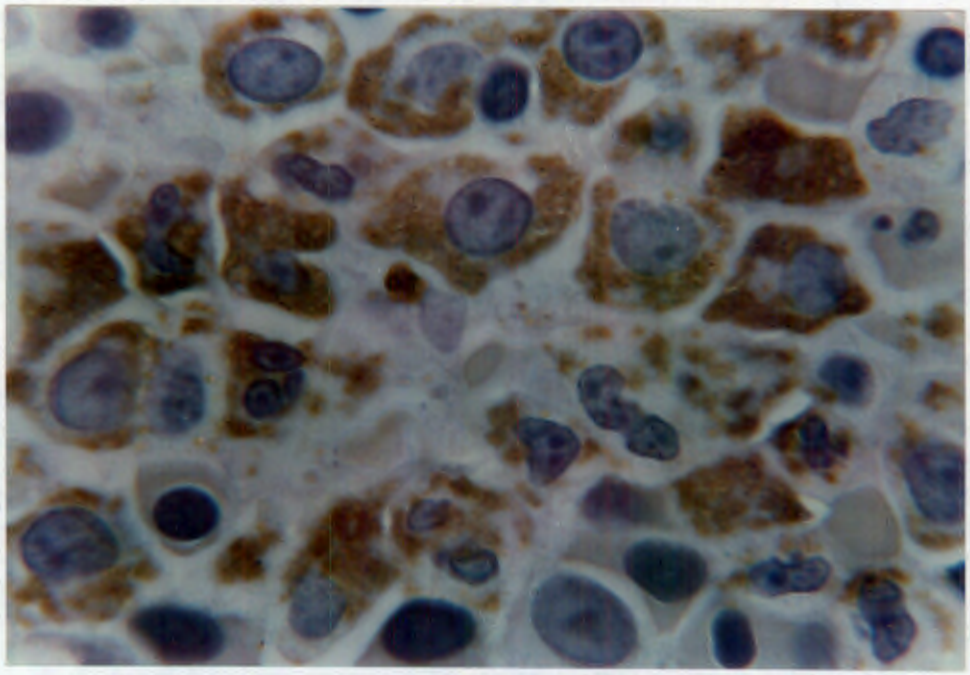
A - aspirate, H - histology, [AH] - aspirate and histology, Cellular - cellularity, DysE - dyserythropoiesis, DysG - dysgranulopoiesis, DysM - dysmegakaryopoiesis, PS - pathological sideroblasts, RS - ring sideroblasts, - not present, + present, P - promyelocytes.

One biopsy [No 6] could not be assessed because of extensive marrow necrosis.

**CYTOCHEMICAL FINDINGS**

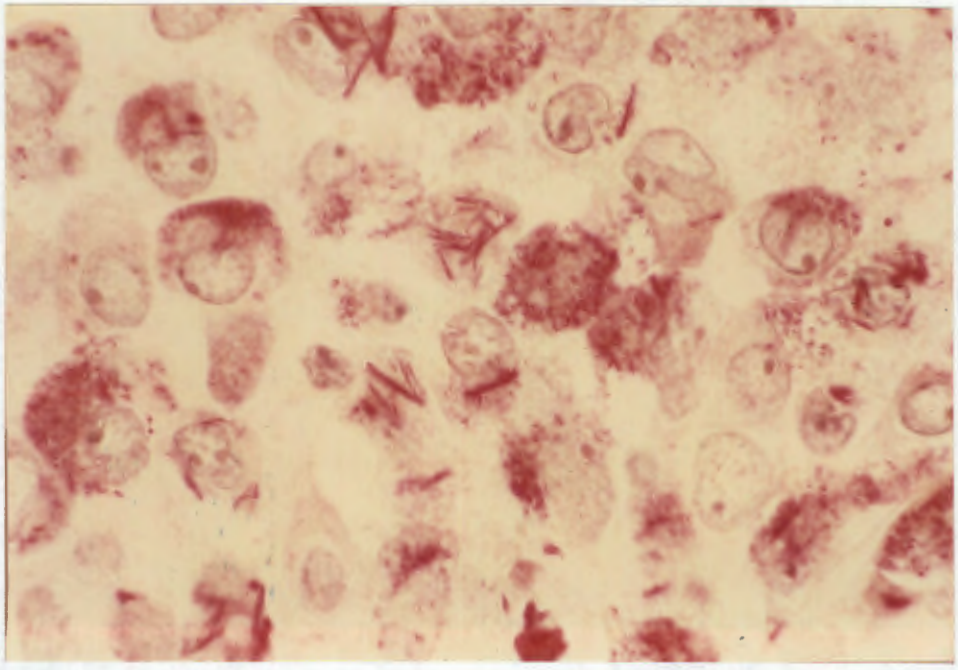
A diagnosis of ANLL was possible in 7 patients, [No's 2, 4, 5, 7, 11, 14, 16] who all showed more than 3% myeloperoxidase positivity in their blasts in the biopsy sections [see Table 19]. Two cases of M3 showed strong [more than 95%] positivity for myeloperoxidase in promyelocytes [Fig.24].

**Figure 24: Myeloperoxidase cytochemistry from a case of APL showing coarse positivity in hypergranular promyelocytes. [x400] GMA**



In case no. 8 the chloroacetate esterase showed the presence of numerous Auer rods [Fig.25].

**Figure 25: Chloroacetate esterase cytochemistry from a case of APL showing coarse positivity and Auer rods in some of the promyeloctes. [x400] GMA**



Myeloperoxidase cytochemistry in patient 3 was negative [less than 3%] on both the aspirate and marrow sections.

In four out of six negative for myeloperoxidase on plastic sections patients [case no's 9, 10, 12, 13], the chloroacetate esterase showed very strong positivity in blasts, thus supporting a diagnosis of ANLL. In the remaining two negative cases, the diagnosis of ANLL had to be aided by immunophenotyping [No's 3, 15]. In the single example of M4E [No 14] chloroacetate esterase stain showed the expected abnormal positivity in eosinophils [Fig.8].

In six out of fifteen individuals reticulum was markedly increased [more than 1+ in grading]. In all four discrepant cases [No's 2, 10, 11, 15] a significantly increased reticulum was found on biopsy [at least 2+ on a grading scale].

An increase in the number of AL-RC was observed in 12 ANLL individuals.

Prussian blue showed only marginal differences in the amount of iron present in marrow aspirate particles and marrow sections. Case No 6 could not be assessed as the marrow was markedly necrotic.

**Table 19: Summary of cytochemical findings**

| Patient | Peroxidase | | CAE | | PAS | | ANBE | | Ac.Phos | | A1.Phos | | Pruss.B | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | A | H | A | H | A | H | A | H | A | H | A | H | A | H |
| 1. | 100%P | 100%P | ND | 80%P | ND | ND | - | - | ND | - | ND | 2+ | ND | 0/6 |
| 2. | 99% | 80% | ND | ND | ND | - | - | - | - | - | ND | 2+ | ND | 3/6 |
| 3. | - | <1% | ND | - | ND | - | - | - | - | - | ND | 1+ | ND | 0/6 |
| 4. | 24% | 15% | ND | 30% | ND | ND | - | - | - | - | ND | 1+ | 1/6 | 1/6 |
| 5. | 87% | 20% | - | - | ND | - | ND | - | - | ND | ND | ND | 3/6 | 3/6 |
| 6. | ------ | ------ | ------ | ------ | ------ | ------ | ------ | ------ | ------ | ------ | ------ | ------ | ------ | ------ |
| 7. | 97% | 40% | ND | 20% | ND | - | ND | ND | ND | +R | ND | N | ND | 1/6 |
| 8. | 98%P | 95%P | ND | 80%P | ND | - | - | - | - | +R | ND | 1+ | 2/6 | 2/6 |
| 9. | 99% | - | ND | 80% | ND | - | ND | - | ND | - | ND | N | 3/6 | 3/6 |
| 10. | 3% | - | ND | 80% | ND | - | - | - | ND | - | ND | 3+ | ND | 4/6 |
| 11. | 83% | >3% | ND | - | ND | - | - | - | 65%NS | - | ND | 2+ | 2/6 | 3/6 |
| 12. | 88% | - | ND | 80% | ND | - | - | - | ND | - | ND | 1+ | ND | 1/6 |
| 13. | 90% | - | ND | 80% | ND | - | - | - | - | - | ND | 2+ | 2/6 | 1/6 |
| 14. | 60% | 30% | ND | 30% Eo+ | ND | 80% | - | - | - | - | ND | 1+ | 1/6 | 1/6 |
| 15. | 92% | - | ND | - | ND | - | - | - | - | - | ND | 2+ | 2/6 | 2/6 |
| 16. | ND | 95%NS | ND | 10% | ND | - | ND | - | ND | - | ND | 3+ | 3/6 | 2/6 |

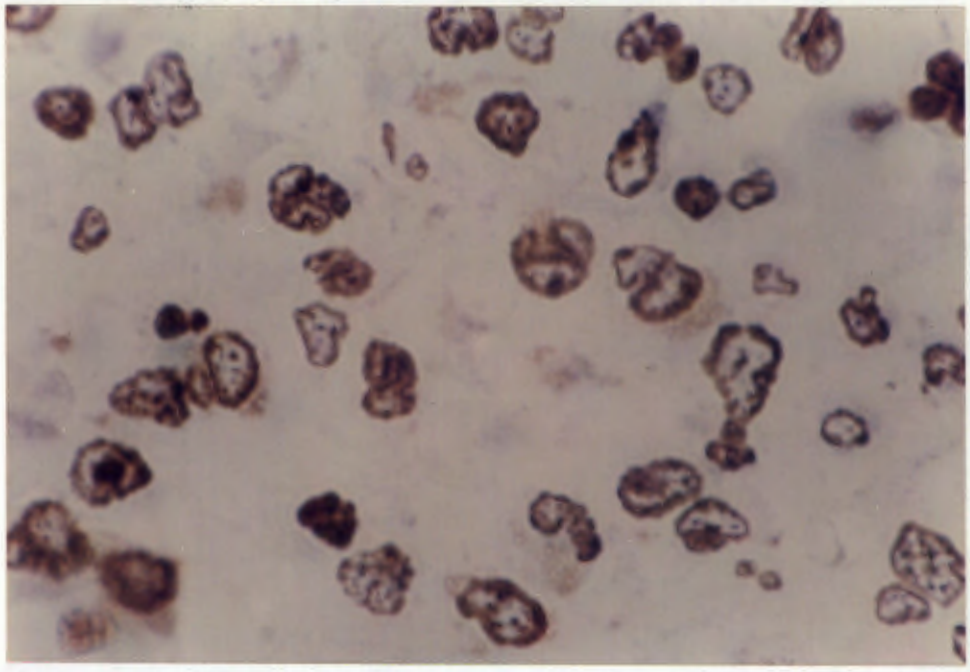
**Abbreviations:**

A - aspirate, H - histology, CAE - chloroacetate esterase, PAS - periodic acid Schiff, Ac.Phos - acid phosphatase, ANBE - alpha-naphthyl butyrate esterase, Al.Phos - alkaline phosphatase, Pruss.B - Prussian blue, PS - pathological sideroblasts, RS - ring sideroblasts, B - blasts, - negative, + positive, P - promyelocytes, R - reticulum cells, Eo - eosinophils, NS - non specific, ND - not done.

**IMMUNOPHENOTYPING**

Immunophenotyping with myeloid antibodies [anti-MPO, CD 13 and CD 33] was performed in four selected patients [No's 3, 11, 14, 15] by using the double-conjugate indirect peroxidase method (99). Anti-MPO antibody [Fig.26] was found to be a very sensitive and reliable marker and was strongly positive in all tested cases.

**Figure 26: Immunophenotyping with anti - myeloperoxidase antibody from a case of MO ANLL showing strong perinuclear myeloperoxidase positivity. [x1000] GMA**



Positivity with CD 13 and CD 33 was achieved in only two cases, after overnight incubation with primary antibody [No's 3, 15] [Fig.27, 28].

**Figure 27: Immunophenotyping with CD 13 antibody from a case of M1 ANLL showing perinuclear positivity. [x1000] GMA**

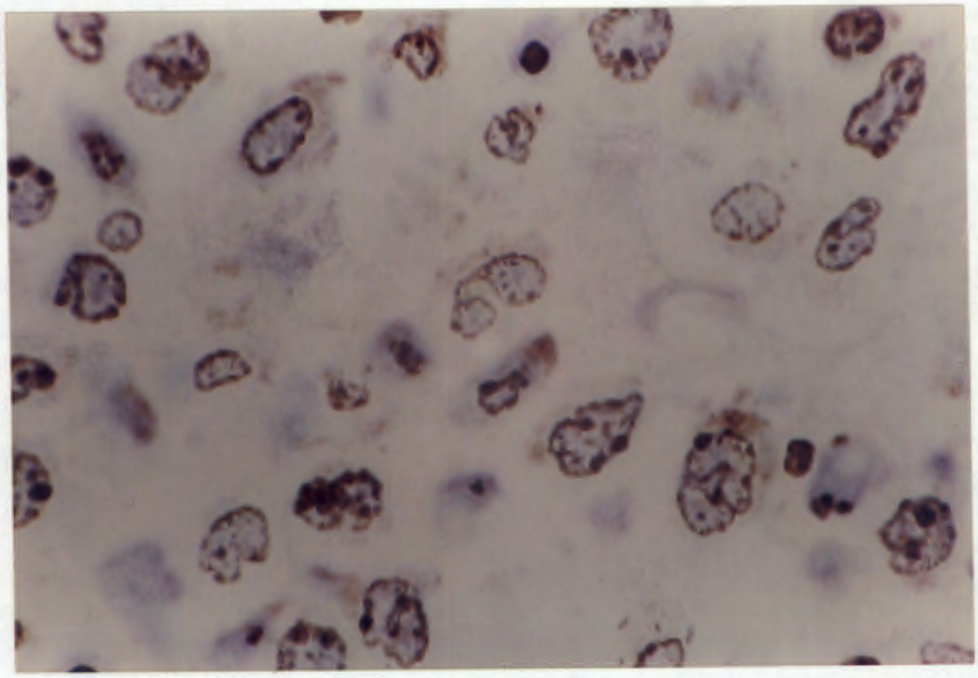
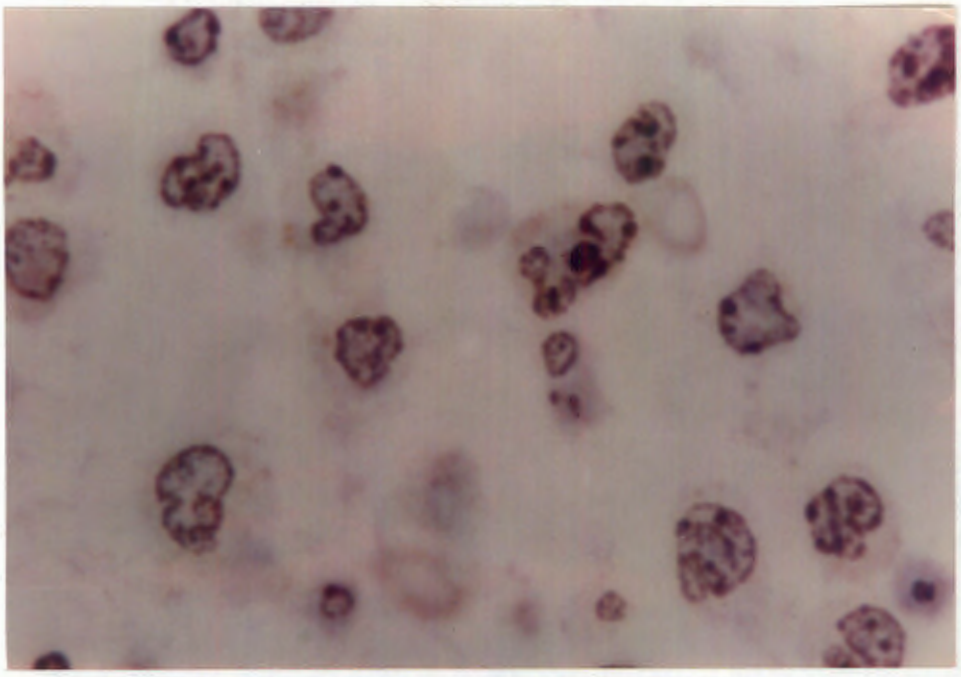


**Figure 28: Immunophenotyping with CD 33 antibody from a case of M1 ANLL showing perinuclear positivity. [x1000] GMA**



## CYTOGENETIC ABNORMALITIES

Cytogenetic abnormalities were detected in eight out of sixteen evaluated patients, with typical t[15;17] for M3 in two patients, inv[16] for M4E in one and t[8;21] for M2 in three cases. Case No. 15 with the t[8;21] abnormality was classified on aspirate as M1, and on biopsy as M2, which is the more likely association (84) [see Table 20].

**Table 20**

| Patient No | Cytogenetic abnormality |
|---|---|
| 1, 8 | t[15;17] |
| 9, 13, 15 | t[8;21] |
| 14 | inv 16 |
| 11 | -17, -22, t[13;15] |
| 16 | 45, XY,-7 |

**FINAL DIAGNOSTIC CONCORDANCE**

Table 21 shows the distribution of the 15 studied cases according to the FAB classification. The largest group of patients [10] were M1 and M2 categories. In 11 cases overall concordance has been shown between marrow sections and aspirate on cytomorphological, cytochemical, and immunogenic criterias.

Differences were noted in the degree of maturation of granulocyte precursors, which was more pronounced in the biopsies. This resulted in a diagnosis of M2 instead of M1 in four out of seven cases of M1 [see Table 21].

**Table 21: Concordance between plastic embedded bone marrow biopsy [BMB] sections and marrow aspirate [BMA]**

**[Total 15 cases]**

| | MO | M1 | M2 | M3 | M4 | M5 | M6 | M7 | [BMA] Total |
|---|---|---|---|---|---|---|---|---|---|
| **MO** | 1 | | | | | | | | 1 |
| **M1** | | 3 | 4 | | | | | | 7 |
| **M2** | | | 3 | | | | | | 3 |
| **M3** | | | | 2 | | | | | 2 |
| **M4** | | | | | 1 | | | | 1 |
| **M5** | | | | | | 0 | | | 0 |
| **M6** | | | | | | | 1 | | 1 |
| **M7** | | | | | | | | 0 | 0 |
| **[BMB] Total** | 1 | 3 | 7 | 2 | 1 | 0 | 1 | 0 | 15 |

**Abbreviations:**

BMB - bone marrow biopsy

BMA - bone marrow aspirate

# CHAPTER FIVE

# DISCUSSION

The classification system of MDS and AL most widely used today is that devised by a collaborating group of French-American-British haematologists. In order to apply the French-American-British [FAB] criteria for the diagnosis of MDS and ANLL [originally designed for blood films and marrow aspirates] to diagnoses based on marrow biopsies, five conditions need to be fulfilled in bone marrow biopsies:

1. Recognition of dysplastic features in all three cell lines.
2. Quantitative evaluation of the percentage of blasts.
3. Quantitative evaluation of the number of sideroblasts.
4. Knowledge of the peripheral blood morphology with blast and monocyte counts.
5. Qualitative and quantitative evaluation of the markers used in defining the subclasses of ANLL:
   - Myeloperoxidase
   - Naphthol-AS chloroacetate esterase [CAE]
   - Alpha-naphthyl butyrate esterase [ANBE]
   - Periodic acid Schiff [PAS]
   - Immunophenotyping

All five criteria will be fulfilled by the use of thin GMA-embedded sections, which give superior preservation of cytological details as well as allowing the application of cytochemical and immunophenotyping techniques.

A unique feature in this study was the use of a -20 $^{o}$C bath with embedding moulds placed at the top [Fig.10], which enabled very effective cooling of the polymerization process; this tended to exceed the boiling point if it was done at room temperature. The boiling point of the embedding mixture was further decreased by application of a vacuum, which was found to be essential for uniform infiltration of the marrow core. All published studies by Henderson (14), Moosavi (21), Islam (33) and Casey (99)

reported polymerization at room temperature or 4 °C, which was found unsuitable in this study as it was complicated by boiling with the resultant formation of air bubbles.

The fixation and dehydration of marrow cores using a 50% concentration of acetone at -20 °C gave good morphologic definition of cells, and processing the biopsies in the cold [-20 °C] enabled excellent preservation of several enzymes useful in diagnosing various haematological malignancies.

Another major advantage of embedding marrow in glycol methacrylate is the relatively short processing time [of 6-8 hours as compared to 36 hours with paraffin embedding] which allows sections to be assessed on the same day as the biopsy is taken.
It is noteworthy that GMA processing of marrow biopsies does not require any special equipment except a microtome, and cutting tissue into 2 microns sections can be done easily, even by an untrained person.

The cost of embedding one marrow core in GMA is about twice that of paraffin, but using this medium is quicker, less complicated, and gives superior morphological details.

**ARCHITECTURAL DISTORTION**

A distortion of topographic distribution of haemopoietic cells in marrow cavity is found in several diseases, but typically in MDS. Distortion of normal marrow architecture was observed in 8 MDS cases, whereas abnormal localization of immature precursors [ALIP] [Fig 6] was noticed on biopsy in four cases with two already being diagnosed as ANLL on GMA sections. Tricot *et al* (1) pointed out the significance of the presence of the abnormal localization of immature precursors [ALIP] which they reported in 28 out of 40 patients. ALIP was also reported in 17 out of 28 MDS patients by Delacretaz *et al* (28).

This feature [together with cellularity] is reported to have not only diagnostic but also prognostic significance (28,32). This means that in some patients [particularly in the MDS group] the FAB category may change after assessment of marrow cavity architectural distortion, and the patient may have a less favourable prognosis, or even overt acute leukaemia (34).

**CELLULARITY**

In general it is believed that bone marrow aspirates do not accurately reflect the marrow cellularity. (13,34) Tricot *et al* (1) suggested that the diagnosis of MDS could not be established on bone marrow smears alone, as these may not be representative.

Similarities in cellularity between aspirates and biopsies were observed in 8 out of 13 MDS cases. Cellularity tends to be higher in biopsies than in aspirates, and this agrees with the study published by De Wolf-Peeters *et al* (30) who found hypercellular marrow in 80% of biopsies, with an increase in reticulin fibrosis in 65% of MDS patients. Paguliuca *et al* (42) reported the presence of marked marrow fibrosis in 10 cases of MDS [median age 66 years], showing trilineage dysplasia with increased megakaryopoiesis and marked reticulin fibrosis. Marked reticulin fibrosis associated with an increased number of megakaryocytes is most likely a result of production [by the latter] of potent polypeptide growth factors, such as platelet-derived growth factor [PDGF] which plays a major role in reticulin formation (2,16,17,109,110). On the contrary, in ANLL patients, megakaryocytes are usually grossly decreased or even absent.

It is reported (106,111) that older patients have a tendency to have greater amounts of reticulin which may explain partially why there were significant differences in cellularity and number of blasts between marrow biopsy and marrow smears in MDS patients [median age 68 years]. Discrepancies between histological and cytological assessment of cellularity in MDS may also result from the patchy distribution of haemopoietic tissue.

The assessment of cellularity in ANLL group in this study showed considerable similarities in all but two cases and in the discrepant cases, differences were small. Such considerable agreement in cellularity and number of blasts in the ANLL group can be related to a lower median age of ANLL patients [44 years], than of the MDS group [68 years]. This observation is similar to findings reported by Islam *et al* (31) who found that assessment of cellularity by aspirate and biopsy were comparable in ANLL.

In summary, cellularity was found to be concordant between aspirate and biopsy in the ANLL group but was significantly discordant in MDS individuals, while no aspirate was obtained in two patients. Thus, in the latter category, biopsy material is essential for diagnosis.

**BLASTS**

Quantification of marrow blasts represents the most important step in the application of the FAB classification of myelodysplastic syndromes and acute leukaemias (7, 8, 9, 10, 11, 23, 29, 40, 76). However, a true estimate of the percentage of blasts in marrow smears can be difficult, because blasts are often in clusters in areas not reached by aspiration or are retained by the reticulin network. Islam *et al* (31) reported that the proportion of haemopoietic cells was more accurately assessed in the sections of marrow cores than the aspirate. In four out of 34 patients, granulocytic precursors were not seen in the marrow aspirate but were easily identified in the biopsies (31).

This problem can be overcome by trephine biopsy, which appears to be more accurate for quantification of the blast population, although identification of blasts in GMA sections is not always easy and requires practice and sometimes application of special stains.

In MDS patients biopsy tended to show a higher number of blasts and also detected blast clusters. This resulted in a change of FAB diagnosis made on aspirate in six

cases of MDS - two patients were diagnosed on marrow biopsy as ANLL against RAEB-t and RA, and 4 were diagnosed on sections as RAEB against RA, RARS, and CMML. Such changes in diagnosis are significant as they have both therapeutic and prognostic implications. This was probably related to the increased reticulum and also to the patchy distribution of blasts in the marrow in MDS.

Discrepancies between aspirate and biopsy in the number of blasts and cellularity was marginal in the ANLL group, with cytology being more sensitive in the detection of blasts. Maturation of granulocytic precursors was more pronounced in the biopsy than on the cytology, which resulted in a diagnosis of M2 instead of M1 in 4 cases.

Thus, biopsy appears to be more accurate in the assessment of haemopoietic cell maturation, quantification of blasts in the MDS group, and is invaluable for diagnosis in cases were no marrow aspirate can be obtained.

**DYSPLASTIC FEATURES**

The detection of dysplastic features in peripheral blood and bone marrow in MDS plays a significant diagnostic role in the FAB classification (2, 4, 9, 32, 49, 70).

The most frequent abnormality observed in marrow biopsies was dysmegakaryopoiesis followed by dysmyelopoiesis and dyserythropoiesis [Table 10].

On GMA-embedded marrow sections dyserythropoiesis [Fig.5] was observed in all 13 cases of MDS, while on aspirate it was not observed in 4 patients [Table 10].

Dysgranulopoiesis was more frequently observed on marrow sections than on aspirate [Table 10]. Use of cytochemistry enabled the detection of cytoplasmic dysplasia [Fig.13], and may play a significant supportive role in the differentiation of cells on marrow sections.

Two patients diagnosed on aspiration as MDS had more than 30% blasts on marrow sections, and with more than 3% positivity for myeloperoxidase and CAE in blasts, could be classified as ANLL. Pseudo Pelgar-Huet anomalies [Fig.6] are reported only in the clonal diseases (for example MDS, ANLL, CML)(48). This abnormality was observed in 10 out of 13 cases on the marrow sections and also in the peripheral blood. These findings are similar to those of Ginnai *et al* (48) who reported that the presence of pseudo Pelgar-Huet anomalies and micromegakaryocytes would enable one to distinguish MDS from other haematological disorders and from normal subjects.

Micromegakaryocytes [Fig.7] were observed in all 13 cases on the marrow sections [Table 10]. Several studies have shown that dysmegakaryopoiesis was frequently present in MDS, and in particular the presence of micromegakaryocytes was limited to MDS and ANLL (1, 28, 29, 32, 34).

The number of peripheral blood blasts and monocytes must also be taken into account in an MDS classification, and in this study four CMML cases were diagnosed mainly on monocyte counts in peripheral blood. Two CMML cases were reclassified on marrow sections as RAEB as a result of an increased number of blasts [5%-20%] in the marrow sections.

In summary, dysplastic features were more readily observed on biopsies than on aspirate, and enabled the use of cytochemistry which markedly improved diagnostic accuracy.

**SPECIAL STAINS**

Careful processing of the specimens at -20 $^{o}$C permitted the performance of cytochemical and immunophenotyping studies which are used in the differentiation of acute myeloid and lymphoid leukaemias.

Myeloperoxidase in this study was positive [more than 3% of blasts positive] on marrow sections in seven cases of ANLL. Four cases were negative, but strong positivity for CAE still enabled the diagnosis of ANLL to be made. In two cases of M3, both myeloperoxidase and CAE were strongly positive, with CAE showing numerous Auer's rods in one of the cases [Fig.25].

In the two subjects negative for MPO and CAE cases the diagnosis had to be supported by myeloid monoclonal antibodies [anti-MPO, CD 13, CD33] [Fig.26, Fig.27, Fig.28].

Two cases of M4 ANLL did not show expected positivity for ANBE on marrow sections, showing only positivity for myeloperoxidase and CAE. In these individuals the diagnosis of M4 was supported by a significant morphologic monocytic component, easily recognizable on GMA marrow sections [Fig.3], and also by abnormal looking monocytes in the peripheral blood which accounted for more than 16 $x10^9$ /L of all cells [case No.4], still making the diagnosis of M4, despite the negative ANBE stain in marrow sections.

A high level of serum lysosyme would be another feature supportive of the diagnosis (8,11). In a second case of M4-M4E [case no. 14], GMA-embedded sections showed 20% eosinophils with positivity for CAE [Fig.8].

Sudan Black B cytochemistry is unsuitable for GMA embedded tissues as application of reagents dissolves the sections.

The staining preparation routinely used to demonstrate reticulin fibre network, Gomori's stain, did not appear to be as positive in GMA sections as on paraffin, but it was found that alkaline phosphatase stains could be used as surrogate tests to detect reticulum increase (22) [Fig.20].

The enzyme acid phosphatase is a valuable marker for lymphoid malignancies and helps to distinguish different types of acute and chronic lymphoproliferative

disorders. It is important in characterizing T and non-T ALL (2,70).

Positivity for acid phosphatase was found to be present only in the reticulum cells of some cases of MDS and ANLL [Fig.17] and was very strongly positive in osteoclasts [Fig.16].

The PAS stain was valuable in showing block positivity in RAEB-t transforming to erythroleukaemia [Fig.15].

Prussian blue staining allowed for iron store estimation [Fig.21] and the detection of ring and pathological sideroblasts [Fig.22].

GMA processed marrow also enabled the detection of stainable iron at the surface of undecalcified trabecular bone, [Fig.23] which was observed in some large studies in 2.8% of marrow biopsies (112). Primary or secondary haemochromatosis appear to be the conditions most likely to be associated with this phenomenon, but it was also noted in malignant conditions (112).

In summary, cytochemistry is generally less important in the assessment of MDS patients, except perhaps alkaline phosphatase for assessment of reticulum. The use of myeloid markers did, however, allow better recognition in some cases of MDS, and uneven cytoplasmic staining was an indication of the dysplastic maturation of myeloid precursors [Fig.13].

Cytochemistry, because of cold processing, excellently preserved enzymatic activity in GMA sections, played a crucial role in the diagnosis and differentiation of AL, also allowing the diagnosis of ANLL in one case, in which no aspirate could be obtained.

**IMMUNOPHENOTYPING**

Immunophenotyping was the most difficult part of this study, requiring very laborious and careful processing. Theoretically, plastic embedding should be superior to frozen and paraffin sections for immunophenotyping through better antigenic and morphologic preservation. In practice, water-soluble plastics like GMA apparently bind to the amino-terminal portions of proteins during polymerization and hence mask antigenic epitopes (13,99).

Casey *et al* (99) reported that the addition of 5% methyl benzoate to the GMA monomer infiltration solution protects antigens from being masked by GMA during polymerization but preserves antigens only in acetone fixed tissues.

He also reported that storage of marrow blocks or sections at -20 $^{o}$C even for several months, did not alter immunoreactivity. This was also our experience.

Immunophenotyping was performed by the double-conjugated indirect peroxidase method. The alkaline phosphatase-antialkaline phosphatase [APAAP] technique was also attempted but did not show the positivity expected in GMA embedded marrow sections, although Casey *et al* (99) reported satisfactory results using this method. As GMA marrow sections have preserved endogenous peroxidase activity, the APAAP method could be most suitable for bone marrow immunophenotyping as no blocking procedure of endogenous peroxidase is required (113). This is an advantage since blocking procedures may denature many of the antigens. The red reaction product of the immuno-alkaline phosphatase procedure is very easy to recognize and contrasts strongly with the haematoxylin counterstain (97).

Beckstead *et al* (23) demonstrated reactivity of megakaryocytic antigens [GPIIa, GPIIb, and the IIb-IIIa complex; granule membrane protein 140] in GMA embedded marrow sections with the use of the double-conjugated indirect peroxidase method.

Casey *et al* (99) reported that a wide variety of antigens can be detected in plastic sections without trypsinisation; pan-B [CD19, CD22,], pan-T [CD7, CD3, CD5, CD2,], T-subsets [CD4, CD8, CD1, CD25] as well as surface immunoglobulin, markers for myeloid antigens [CD13, CD33,] and monocytic antigens [CD14, CD15]. By using this method we were able to show reactivity with anti-MPO, CD13 and CD33, with the last two antibodies reacting only after modification of the method by incubating overnight with primary antibody. In our study we could not reproduce the reactivity of antigens with monocytic antibodies as reported by Casey *et al* despite very stringent application of the method.

The MPO antibody which allows recognition of myeloperoxidase positive cells was found to be reliable and most sensitive in the identification of cells of myeloid origin [Fig.26]. CD13 and CD33 positivity was achieved only after overnight incubation at 4 $^{o}$C with the primary antibody, which considerably extends processing time [Fig.27, 28]. Together these three monoclonal antibodies allow for diagnosis of more than 95% of ANLL, (88, 91) which make them very valuable in this setting, particularly in cases where no aspirate can be obtained.

# CHAPTER SIX

## SUMMARY

In the MDS group of patients, final morphological agreement in diagnosis was observed in only 7 out of 13 cases, with significant changes of diagnosis on marrow sections in 6 patients, implying that biopsy, with its more accurate blast count, is more precise and accurate in the classification of MDS, and also allows a diagnosis to be made in those cases in which marrow aspirates cannot be obtained.

In the ANLL group, combined histological, cytological and immunological marrow aspirate classification was in agreement in 11 out of 15 cases of ANLL, with marrow sections showing more accurately the maturation present in myeloid cells, resulting in a diagnosis of M2 instead of M1 in 4 ANLL cases.

# CHAPTER SEVEN

# CONCLUSIONS

This study showed that the FAB Cooperative Group criteria, originally designed for marrow aspirates, can be applied to thin GMA embedded marrow biopsies and can allow the diagnosis of MDS or ANLL in cases where aspiration is inadequate. These criteria, when applied to marrow sections, allow for more precise and accurate differentiation of leukaemic sub-types.

The maximal accuracy can, however, be obtained only by analysis of morphological, cytochemical, immunological, karyotypic and, in some cases, ultrastructural investigations.

Another major advantage of embedding marrow in glycol methacrylate is the relatively short processing time of 6-8 hours [as compared to 36 hours with paraffin embedding] which allows sections to be assessed on the same day as the biopsy is taken.

The simplicity, short processing time, reproducibility, excellent morphology, cytochemical and immunophenotyping staining makes this method highly suitable for routine work in the haematology laboratory.

# REFERENCES


1. Tricot G, De Wolf - Peeters C, Hendrickx B, Verwilghen RL. Bone marrow histology in myelodysplastic syndromes. *Br J Haematol* 1984;**57**: 423.

2. Hoffbrand AV, Lewis SM. Postgraduate Haematology. Section 13 , Cytogenetics and leukaemogenesis, pp 358 - 378, Section 14, Acute leukaemia, pp 379 - 417, Section 17, The myelodysplastic syndrome, pp 463 - 473 Third edition 1989 Churchill Livingstone (Edinburgh, London, Melbourne and New York).

3. Luzzatto L, Colonna F, Reily I. The "n-1" model for myelodysplastic syndromes. *Leuk Res* 1992;**16**: 57.

4. Williams WJ, Bentler E, Ersler A, Lichtman MA. Haematology. Part II, Section 1, Structure and function of haemopoietic organs, pp 37- 47, Part III, Section 1, The concept of the haemopoietic stem cell, pp 129 - 147, Section 2, Chapter 17, Classification and clinical manifestation of the haemopoietic stem cell disorders, pp 148 - 157, Section 4, Chapter 19, Preleukaemia and oligoblastic leukaemia, pp 175 - 185, Section 5, Chapter 26, Acute myelogenous leukaemia, pp 251-272 Fourth edition 1991 McGraw - Hill Publishing Company (London, New York, Tokyo).

5. Verwilghen RL, Boogaerts MA. Myelodysplastic syndrome. *Blood Rev* 1987;**1**: 34.

6. Schumacher HR. Acute leukaemia. First edition. 1990. Igaku - Shoin (New York, Tokyo).

7. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Proposal for the recognition of minimally differentiated acute myeloid leukaemia. (AML-MO). *Br J Haematol* 1991;**78**: 325.

8. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Proposals for the classification of the acute leukaemia. *Br J Haematol* 1976;**33**: 451.

9. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR and Sultan C. Proposals for the classification of the myelodysplastic syndromes. *Br J Haematol* 1982;**51**: 189.

10. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR and Sultan C. Criteria for the diagnosis of acute leukaemia of megakaryocyte lineage (M7). A report of the French - American - British Cooperative Group. *Ann Inter Med* 1985;**103**: 460.

11. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR and Sultan C. A report of the French - American - British Cooperative Group. Proposed revised criteria for the classfication of acute myeloid leukaemia. *Ann Inter Med* 1985;**103**: 626.

12. Bennet JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR and Sultan C. (FAB cooperative group). A variant form of hypergranular promyelocytic leukaemia. (M3) *Br J Haematol* 1980;**44**: 169.

13. Islam A, Frish B, Henderson ES. Plastic embedded core biopsy: a complementary approach to bone marrow aspiration for diagnosing acute myeloid leukaemia. *J Clin Pathol* 1989;**42**: 300.

14. Henderson ES, Islam A. The role of bone marrow biopsy in haematological disorders with special reference to plastic embedded material. *Haematology Rev* 1990;**4**: 247.

15. Islam A and Henderson ES. Value of long - core biopsy in the detection of discrete bone marrow lesions. *Histopathology* 1988;**12**: 641.

16. McCarthy DM. Fibrosis of the bone marrow: content and causes. Annotation. *Br J Haematol* 1985;**59**: 1.

17. Manoharan A, Horsley R and Pitney WR. The reticulin content of bone marrow in acute leukaemia in adults. *Br J Haematol* 1979;**43**: 185.

18. Hall PA, Lindeman R, Butler MG, Amess JAL, D'Ardenne AJ. Demonstration of lymphoid antigens in decalcified bone marrow trephines. *J Clin Pathol* 1987;**40**: 870.

19. Athanasou NA, Quinn J, Heryet A, Woods CG, McGee JO'D. Effect of decalcification agents on immunoreactivity of cellular antigens. *J Clin Pathol* 1987;**40**: 874.

20. Beckstead JH, Stenberg P, McEver RP, Shuman MA, Bainton DF. Immunohistochemical localization of membrane and alpha - granule proteins in human megakaryocytes: application to plastic - embedded bone marrow biopsy specimens. *Blood* 1986;**67**: 285.

21. Moosavi H, Lichtman MA, Donnelly JA, Churukain CJ. Plastic - embedded human marrow biopsy specimens. *Arch Pathol Lab Med* 1981;**105**: 269.

22. Beckstead JH, Halverson PS, Ries CA, Bainton DF. Enzyme histochemistry and immunohistochemistry on biopsy specimens of pathologic human bone marrow. *Blood* 1981;**57**: 1088.

23. Beckstead JH. The bone marrow biopsy, a diagnostic strategy. *Arch Pathol Lab Med* 1986;**110**: 175.

24. Henderson ES, Islam A. Glycol methacrylate embedding for light microscopy I: enzyme histochemistry on semithin sections of undecalcified marrow cores. *J Clin Pathol* 1987;**40**: 1194.

25. Frisch B, Islam A. Plastic embedding in routine histology I: preparation of semi - thin sections of undecalcified marrow cores. *Histopathology* 1985;**9**: 1263.

26. Hott M, Marie PJ. Glycol methacrylate as an embedding medium for bone. *Stain Technol* 1987;**62**: 51.

27. Thiele J, Quitmann H, Wagner S, Fisher R. Dysmegakaryopoiesis in myelodysplastic syndromes: an immunomorphometric study of bone marrow trephine biopsy specimens. *J Clin Pathol* 1991;**44**: 300.

28. Delacretaz F, Schmidt PM, Piguet D, Bachmann F, Costa J. Histopathology of myelodysplastic syndromes : the FAB classification (proposals) applied to bone marrow biopsy. *Am J Clin Pathol* 1987;**87**: 180.

29. Frisch B, Bartl R. Bone marrow histology in myelodysplastic syndromes. *Scand J Haematol* 1986;**36**: 21.

30. De Wolf - Peeters C, Tricot G, Verwilghen RL, Vlietinck R. Bone marrow histology in myelodysplastic syndromes. *Br J Haematol* 1984;**58**: 217.

31. Islam A, Catovsky D, Goldman JM, Galton DAG. Bone marrow biopsy change in acute myeloid leukaemia 1: observation before chemotherapy. *Histopathology* 1985;**9**: 939.

32. Canizo MC, Gomis F, Rios A, Ruiz C, Sanz G, Sanz MA, San Miguel JF, Torrabadella M, Vallespi T. Bone marrow biopsy in myelodysplastic syndromes: morphological characteristics and contribution to the study of prognostic factors. *Br J Haematol* 1990;**75**: 26.

33. Islam A, Frisch B, Henderson ES. Value of processing bone marrow biopsies in plastic for haematological diagnosis. *Haematology Rev* 1990;**4**: 151.

34. Lambertenghi - Deliliers G, Annaloro C. Histological diagnosis in myelodysplastic syndromes. *Haematology Rev* 1989;**3**: 109.

35. Lazzaro B, Lumb G, Munger R. Antigen localization in immunoperoxidase - stained plastic - embedded soft tissues. *Human Pathol* 1988;**19**: 902.

36. Davey FR, Elghetany MT, Kurec AS. Immunophenotyping of haematological neoplasms in paraffin - embedded tissue sections. *Am J Clin Pathol* 1990;**93**: 17.

37. More NS, Rowden G, Sacher RA. Plastic embedded specimens for evaluation of bone marrow. Top Rev Haematol PSG Right Bristol 2 1982;2: 1.

38. Bain BJ. Leukaemia Diagnosis. A guide to the FAB classification. First edition 1990 J B Lippincott Company (Philadelphia).

39. Bagby GC. The preleukemic syndrome (Hematopoietic dysplasia). *Blood Rev* 1988;**2**: 194.

40. Bartl R, Frisch B, Baugart R. Morphologic classification of the myelodysplastic syndromes (MDS): combined utilization of bone marrow aspirates and trephine biopsies. *Leuk Res* 1992;**16**: 15.

41. Gladson C. Faramarz. Hypocellular bone marrow with increased blasts. *Am J Haematol* 1986;**21**: 15.

42. Pagulica A, Layton DM, Gordon S, Green PJ, Mufti GJ. Myelofibrosis in primary myelodysplastic syndromes: a clinical - morphological study of 10 cases. *Br J Haematol* 1988;**71**: 499.

43. Hamblin TJ. The treatment of acute myeloid leukaemia preceded by the myelodysplastic syndrome. *Leuk Res* 1992;**16**: 101.

44. Hamblin TJ. Clinical features of MDS. *Leuk Res* 1992;**16**: 89.

45. Coutinho LH, Geary CG, Change J, Harrison C, Testa NG. Functional studies of marrow haemopoietic and stromal cells in the myelodysplastic syndrome. *Br J Haematol* 1990;**75**: 16.

46. Hasle H, Jacobsen BB and Pedersen NT. Myelodysplastic syndromes in childhood: a population based study of nine cases. *Br J Haematol* 1992;**81**: 495.

47. Chisholm M, McMullin MF, Hows JM. Congenital myelodysplasia: a new described disease entity? *Br J Haematol* 1991;**79**: 340.

48. Ginnai I, Ichimaru M, Kuriyama K, Matsuo T, Tomonaga M. Diagnostic significance of detecting pseudo Pelger - Huet anomalies and micro - megakaryocytes in myelodysplastic syndrome. *Br J Haematol* 1986;**63**: 665.

49. Frish B, Bartel R. Atlas of bone marrow pathology. Primary and secondary myelodysplastic syndromes, pp 67 - 80, Acute leukaemia, pp 81 - 92. First edition 1990 Kluwer Academic (Dordrecht, Boston).

50. Mufti GJ. Chromosomal deletions in the myelodysplastic syndrome. *Leuk Res* 1992;**16**: 35.

51. White AD, Culligan DJ, Hoy TG and Jacobs A. Extended cytogenetic follow - up of patients with myelodysplastic syndrome (MDS). *Br J Haematol* 1992;**81**: 499.

52. Heim S. Cytogenetic findings in primary and secondary MDS. *Leuk Res* 1992;**16**: 43.

53. Jacobs A. Gene mutations in myelodysplasia. *Leuk Res* 1992;**16**: 47.

54. Hoffbrand AV. Recent advances in haematology. Section 9, Viruses, oncogenes and leukaemia, pp 221 - 237, Section 10, The myelodysplastic syndrome and preleukaemia, pp 239 - 267 Number Four 1985 Churchill Livingstone (Edinburgh, London, Melbourne and New York).

55. Hamblin TJ and Oscier DG. The myelodysplastic syndrome - a practical guide. *Haematol Oncol* 1987;**5**: 19.

56. Copplestone JA, Figes A, Hamblin TJ, Mufti GJ, Oscier DG. Immunological abnormalities in myelodysplastic syndromes. *Br J Haematol* 1986;**63**: 143.

57. Bennett JM, Chuang C, Varela BL, Woll JE. Modifications in the classification of primary myelodysplastic syndromes: the addition of a scoring system. *Haematol Oncol* 1985;**3**: 55.

58. Sanz FG and Sanz MA. Prognostic factors in myelodysplastic syndromes. *Leuk Res* 1992;**16**: 77.

59. Tricot GJ. Prognostic factors in the myelodysplastic syndromes. *Leuk Res* 1992;**16**: 109.

60. Cosson A, Esteinne MH, Fenaux P, Lai JL, Lepelley P, Preudhomme C, Zandecki M. Prognostic value of dysmyelopoietic features in de novo acute myeloid leukaemia : a report on 132 patients. *Clin Lab Haematol* 1990;**12**: 57.

61. Greenberg PL. Treatment of myelodysplastic syndromes. *Blood Rev* 1991;**5**: 42.

62. Tricot GJ. Complications and treatment of the myelodysplastic syndromes. *Leuk Res* 1992;**16**: 117.

63. Hast R and Hellstrom E. Therapeutic aspects of myelodysplastic syndromes in chronic phase. *Leuk Res* 1992;**16**: 95.

64. Bain B, Catovsky D. Current concepts in haematology 2: classification of acute leukaemia. *J Clin Pathol* 1990;**43**: 882.

65. Gale RP. Hybrid acute leukaemia. Annotation. *Br J Haematol* 1987;**65**: 261.

66. Hoffbrand AV, Leber BF, Browett PJ and Norton JD. Mixed acute leukaemia. *Blood Rev* 1988;**2**: 9.

67. Stass A and Mirro J. Lineage heterogeneity in acute leukaemia: acute mixed - lineage leukaemia and lineage switch. *Clin Haematol* 1986;**15**: 811.

68. Pedersen - Bjergaard J. Radiotherapy and chemotherapy induced myelodysplasia and acute myeloid leukaemia. *Leuk Res 1992*;**16**: 61.

69. Schumacher HR, Shrit MA, Kowal - Vern A, Dizikes G, Radvany RM, Le Beau MM. Acute leukaemia and related entities. *Arch Pathol Lab Med* 1991;**115**: 331.

70. Hall R, Malia RG. Medical laboratory. Haematology. Section 13, Leukaemia and the proliferative disorders, pp 419 - 500 Second edition 1991 Butterworth - Heinemann Ltd (Oxford).

71. Lindquist R, Nilsson B, Eklund G, Gahrton G. Acute leukaemia in professional drivers exposed to gasoline and diesel. *Eur J Haematol* 1991;**47**: 98.

72. Brandt L. Exposure to organic solvents and risk of haematological malignancies. *Leuk Res* 1992;**16**: 67.

73. Coltman CA, Dahlberg S. Treatment - related leukaemia. *N Eng J Med* 1990;**322**: 52.

74. Pedersen - Bjergaard J, Specht L, Larsen OS, Ersboll J, Struck J, Hansen MM, Hansen HH, Nissen NI. Risk of therapy - related leukaemia and preleukaemia after Hodgkin's disease. *Lancet* 1987;**2**: 83

75. Brito - Babapulle F, Pullon H, Layton DM, Etches A, Huxtable A, Mangi M, Bellingham AJ, Mufti GJ. Clinicopathological features of acute undifferentiated leukaemia with a stem cell phenotype. *Br J Haematol* 1990;**76**: 210.

76. A participants in the Second MIC Cooperative Study Group: Bennett JM, Berger R, Catovsky D, Flandrin G, Foon KA, Gralnick HR, Griffin JD, Hagemeijer A, Mecucci C, Minowada J, Mitelman F, Preud'homme JL, Snadberg AA, Van den Berghe H. Morphologic, immunologic and cytogenetic (MIC) working classification of the acute myeloid leukaemia. *Br J Haematol* 1988;**68**: 487.

77. Bitter MA, Golomb HM, Larson RA, Le Beau MM, Rowley JD, Vardiman JW. Association of an inversion of chromosome 16 with abnormal marrow eosinophils in acute myelomonocytic leukaemia. *N Eng J Med* 1983;**309**: 630.

78. Boucheix C, Chastang C, Debre P, Leblond V, Merle -Beral H, Michel A, Nguyen Cong Duc L. Diagnostic and prognostic significance of myelomonocytic cell surface antigens in acute myeloid leukaemia. *Br J Haematol* 1989;**73**: 323.

79. Breton - Gorius J, Vanhaeke D, Pryzwansky KB, Guichard J, Tabilo A, Vainchenker W and Carmel R. Simultaneous detection of membrane markers with monoclonal antibodies and peroxidatic activities in leukaemia: ultrastructural analysis using a new method of fixation preserving the platelet peroxidase. *Br J Haematol* 1984;**58**: 447.

80. Hanson CA, Gajl - Peczalska KJ, Parkin JL, Brunning RD. Immunophenotyping of acute myeloid leukaemia using monoclonal antibodies and the alkaline phosphatase - antialkaline phosphatase technique. *Blood* 1987;**70**: 83.

81. Buccheri V, Shetty V, Yoshida R, Morilla E, Catovsky D. The role of an anti - myeloperoxidase antibody in the diagnosis and classification of acute leukaemia: a comparison with light and electron microscopy cytochemistry. *Br J Haematol* 1992;**80**: 62.

82. Geller RB, Zahurak M, Hurwitz GA, Burke PJ, Karp JE, Piantadosi S, Civin CI. Prognostic importance of immunophenotyping in adults with acute myelocytic leukaemia: the significance of the stem - cell glycoprotein CD34(MY10). *Br J Haematol* 1990;**76**: 340.

83. Catovsky D, Foroni L, Matutes E, Morilla R, Pombo de Oliveira M. The role of ultrastructural cytochemistry and monoclonal antibodies in clarifying the nature of undifferentiated cells in acute leukaemia. *Br J Haematol* 1988;**69**: 205.

84. Swirsky DM, Matthews JG, Flemans RJ, Rees JKH, Hayhoe FGJ, Li YS. 8;21 translocation in acute granulocytic leukaemia: cytological, cytochemical and clinical features. *Br J Haematol* 1984;**56**: 199.

85. Tantravahi R, Schwenn M, Henkle C, Leavitt PR, Griffin JD, Nell M, Weinstein HJ. A pericentric inversion of chromosome 16 is associated with dysplastic marrow eosinophils in acute myelomonocytic leukaemia. *Blood* 1984;**63**: 800.

86. Hoffbrand AV. Recent advances in haematology. Section 8, Acute leukaemia, pp 161 - 182 Number Three 1982 Churchill Livingstone (Edinburgh, London, Melbourne and New York).

87. Kurec AS, Cruz VE, Barret D, Mason D and Davey FR. Immunophenotyping of acute leukaemia using paraffin - embedded tissue sections. *Am J Clin Pathol* 1990;**93**: 502.

88. Matutes E, De Oliveira MP, Foroni L, Morilla R and Catovsky D. The role of ultrastructural cytochemistry and monoclonal antibodies in clarifying the nature of undifferentiated cells in acute leukaemia. *Br J Haematol* 1988;**69**: 205.

89. Neame PB, Soamboonsrup P, Browman GP, Meyer RM, Benger A, Edwin W, Wilson C, Walker IR, Saeed N and McBride JA. Classifying acute leukaemia by immunophenotyping: a combined FAB - immunologic classification of AML. *Blood* 1986;**68**: 1355.

90. Sacchi S, Marco M, Vecchi A, Morselli S, Longo R, Grande A, Toreli U. The use of the alkaline phosphatase - antialkaline phosphatase technique for immunophenotyping acute myeloid leukaemia. *Acta Haematol* 1991;**85**: 6.

91. Storr J, Dolan G, Coustan - Smith E, Barens D, Reilly JT. Value of monoclonal anti - myeloperoxidase (MPO7) for diagnosing acute leukaemia. *J Clin Pathol* 1990;**43**: 847.

92. Jain NC, Cox C and Bennett JM. Auer rods in the acute myeloid leukemias: frequency and methods of demonstration. *Hematol Oncol* 1987;**5**: 197.

93. Wilkins BS and Jones DB. The use of immunohistochemical techniques with fixed, decalcified, wax - embedded bone marrow trephine biopsies. *Haematol Rev* 1989;**3**: 149.

94. Brown BA. Haematology: principles and procedures. Section 5, Special haematology procedures, pp 173 - 183 Third edition 1980 Lea & Febiger (Philadelphia).

95. Dacie JV, Lewis SM. Practical Haematology. Section 10, Leukocyte cytochemical and immunological techniques, pp 125 - 155 , Section 11, Bone marrow biopsy, pp 157 - 173 Seventh edition 1991 Churchill Livingstone (Edinburgh, London, Melbourne and New York).

96. Bain BJ. Blood cells. A practical guide. Section 7, Important supplementary tests, pp 169 - 186, Section 8, Interpretation - white cells, pp 187 - 253. First edition 1989 J B Lippincott Company (Philadelphia).

97. Moir DJ, Abdulaziz Z, Knight PM, Mason DY, Ghosh AK. Immunoenzymatic staining of haematological samples with monoclonal antibodies. *Br J Haematol* 1983;**55**: 395.

98. Argyle JC, Benjamin DR, Hammond D, Lampkin B. Acute nonlymophocytic leukaemia of childhood. *Cancer* 1989;**63**: 295.

99. Casey TT, Collins RD, Cousar JB. A simplified plastic embedding and immunohistologic technique for immunophenotypic analysis of human haemopoietic and lymphoid tissues. *Am J Path* 1988;**131**: 183.

100. Foon KA, Gale RP. Therapy of acute myelogenous leukaemia. *Blood Rev* 1992;**6**: 15.

101. Lo Coco F, Avvisati G, Diverio D, Petti MC, Alcalay M, Pandolfi PP, Zangrilli D, Biodi A, Rambaldi A, Moleti ML, Mandelli F and Pelicci PG. Molecular evaluation of response to all - trans - retinoic acid therapy in patients with acute promyelocytic leukaemia. *Blood* 1991;**77**: 1657.

102. Stone RM, Maguire M, Goldberg MA, Antin JH, Rosenthal DS and Mayer RJ. Complete remission in acute promyelocytic leukaemia despite persistence of abnormal bone marrow promyelocytes during induction therapy: experience in 34 patients. *Blood* 1988;**71**: 690.

103. Tallman MS and Hau CK. Reassessing the hemostatic disorder associated with acute promyelocytic leukaemia. *Blood* 1992;**79**: 543.

104. Hayhoe FGJ, Flemans RJ. A colour atlas of haematological cytology. Appendix: staining techniques pp 233 Second edition. 1982. Wolfe Medical Publications Ltd (Netherlands).

105. Hayhoe FGJ, Quaglino D. Haematological cytochemistry. First edition. 1980. Churchill Livingstone (Edinburgh, London, Melbourne and New York).

106. Beckman EN, Brown AW. Normal reticulin level in iliac bone marrow. *Arch Pathol Lab Med* 1990;**114**: 1241.

107. Torrance J, Gale E, Bothwell T. The quantitative estimation of total iron stores in human bone marrow. *J Clin Invest* 1963;**42**: 1076.

108. Dilly SA, Jagger CJ. Bone marrow stromal cell changes in haematological malignancies. *J Clin Pathol* 1990;**43**: 942.

109. Hasselbach H. Idiopathic myelofibrosis: a review. *Eur J Haematol* 1990;**45**: 65.

110. Imbert M, Nguyen D, Sultan C. Myelodysplastic syndromes (MDS) and acute myeloid leukemias (AML) with myelofibrosis. *Leuk Res* 1992;**16**: 51.

111. Weinstein IM. Idiopathic myelofibrosis: Historical review, diagnosis and management. *Blood Rev* 1991;**5**: 98.

112. Cottier H, Egger T, Laeng H, Roethlisberger C. Stainable bone iron in undecalcified, plastic - embedded sections. *Am J Path* 1988;**131**: 344.

113. Cordell JL, Falini B, Erber WN, Ghosh AK, Abdulaziz Z, MacDonald S, Pulford KAF, Stein H, Mason DY. Immunoenzymatic labelling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti - alkaline phosphatase (APAAP) complexes. *J Histochem Cytochem* 1984;**32**: 219.